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Manish Kumar

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**ASSESSMENT OF MOLECULAR ACTION OF DIRECT GATING AND
ALLOSTERIC MODULATORY EFFECTS OF CARISOPRODOL (SOMA®) ON
GABA_A RECEPTORS**

By

Manoj Kumar, B.Pharm.

**Dissertation submitted to the School of Pharmacy at
West Virginia University in partial fulfillment of
the requirements for degree of**

**DOCTOR OF PHILOSOPHY
IN
PHARMACEUTICAL AND PHARMACOLOGICAL SCIENCES**

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**Keywords: GABA_A receptors, GABA_A receptor isoforms, Carisoprodol, muscle
relaxant, drug abuse**

ABSTRACT

**ASSESSMENT OF MOLECULAR ACTION OF DIRECT GATING AND
ALLOSTERIC MODULATORY EFFECTS OF CARISOPRODOL (SOMA®) ON
GABA_A RECEPTORS**

Manoj Kumar, B.Pharm.

Carisoprodol is a centrally acting skeletal muscle relaxant frequently prescribed for the treatment of acute musculoskeletal conditions. Recreational use of carisoprodol is an increasing problem. Recent reports highlight the dangers involved in carisoprodol abuse, including severe withdrawal leading to seizures and death. Indeed, carisoprodol has been placed into schedule IV drug at federal level effective January 11, 2012 considering its alarming abuse rate. Until recently, it was widely accepted that the sedative effects of carisoprodol were due predominantly to its metabolite, meprobamate. Our lab recently concluded, that carisoprodol itself, at low concentrations allosterically modulates and at higher concentrations directly activates γ -aminobutyric acid, type A receptors (GABA_ARs), the predominant inhibitory neurotransmitter receptor in mammalian brain. This may underlie the capacity of carisoprodol to enhance the sedative effects of CNS depressants, contributing to its potential for abuse.

GABA_A receptors are member of the cys-loop receptor family that are heteropentameric ligand gated chloride ion channels and play a critical role in mediating fast inhibition in the brain. A diverse number of GABA_AR subunits as well as their isoforms have been identified including α (1-6), β (1-3), γ (1-3), ρ , δ , ϵ and θ . $\alpha\beta\gamma$ and $\alpha\beta\delta$ receptor (2:2:1 stoichiometry) isoforms are the predominant GABA_ARs in the CNS. $\alpha\beta\gamma$ GABA_ARs generally located within the synapses mediating GABAergic phasic inhibition, whereas $\alpha\beta\delta$ receptors are preferentially targeted to extrasynaptic membranes inducing tonic inhibition. Each GABA_AR subunit is composed of a large extracellular N terminus, four transmembrane helices (TM1–TM4), one extracellular TM2–TM3 loop, two intracellular loops (TM1–TM2 and TM3–TM4), and an extracellular C terminus. TM2 from each subunit line the pore of the channel and gate. We hypothesized that carisoprodol modulates GABA_ARs in a subunit-dependent manner, possibly through a novel site of action, with different amino acid domains contributing to direct gating and allosteric modulatory actions of carisoprodol. We used HEK293 cells, stably and transiently expressing desired GABA_A receptor and took advantage of whole cell patch clamp electrophysiology and site directed mutagenesis techniques to address following specific aims: 1) To identify subunit-dependent influence on direct gating and allosteric enhancement effects of carisoprodol on GABA_ARs; 2) To identify GABA_A receptor subunit domains that confer carisoprodol sensitivity.

Our studies demonstrate direct gating and allosteric modulatory effects of carisoprodol are GABA_A receptor isoforms dependent. Specifically, carisoprodol is most efficacious on $\alpha 1$ containing receptors for allosteric modulatory action and least efficacious on $\alpha 3$ containing receptors for direct gating action. Also, presence or absence of γ subunit in

GABA_AR does not affect carisoprodol direct gating and allosteric modulatory efficacy. Regarding δ containing extrasynaptic receptors, carisoprodol is more efficacious than GABA and potentiated the maximal GABA-gated currents like barbiturates and general anesthetics. Mutagenesis studies showed, transmembrane 4 domains of α subunit are involved in direct gating action of carisoprodol but not in allosteric modulatory action. Mutation of valine at 440 TM4 domain of $\alpha 3$ subunit to corresponding $\alpha 1$ subunit residue leucine 415 significantly increased the direct gating efficacy of carisoprodol without affecting allosteric modulation properties. In corresponding reverse mutation experiments, mutation of $\alpha 1$ subunit residue leucine 415 to valine 440 residue of $\alpha 3$ subunit significantly reduced direct gating efficacy of carisoprodol but not allosteric effects. In physicochemical analysis of TM4 415 residue, polarity and volume of amino acid influenced the direct gating efficacy of carisoprodol. Taken together, our data indicate that carisoprodol interacts with distinct sites to allosterically modulate and directly gate GABA_A receptors. Also, the pharmacological profile of carisoprodol at GABA_A receptors coincide with its therapeutic effects, and carisoprodol's subunit-dependence property may underlie its potential for abuse.

DEDICATION

I want to dedicate my work to my parents, **Nirmla Devi** and **Om Prakash**, for all the support, who stood by me during ups and downs, supported, inspired and encouraged me to go after my goals. I want to thank you with all my heart for everything.

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LIST OF ABBREVIATIONS

GABA	γ -aminobutyric acid
GABA _A R	γ -aminobutyric acid type A receptor
LGIC	Ligand-gated ion channel
CSP	Carisoprodol
BZD	Benzodiazepines
PTX	Picrotoxin
PB	Pentobarbital
CYP2C19	Cytochrome P450 2C19 enzyme
TM	Transmembrane
HEK	Human embryonic kidney
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol-bis (β -aminoethyl ether)
HEPES	N-2-hydroxyethylpiperazine-N-2-etanesulfonicacid N, N, N', N'-tetra acetic acid

CHAPTER I

INTRODUCTION

Carisoprodol (CSP) is a centrally acting muscle relaxant, frequently prescribed to relieve skeletal muscle spasms and associated pain (Toth and Urtis, 2004). Recent evidence confirms carisoprodol abuse leads to several adverse effects, such as psychomotor impairment and severe withdrawal that may predispose to hallucinations, seizures and death (Bramness et al., 2004; Fass, 2010; Zacny and Gutierrez, 2011; Zacny et al., 2012). Carisoprodol abuse has increased rapidly in recent years. Recreational users abuse carisoprodol for its muscle relaxing, anxiolytic and sedating effects, and often mix it with other CNS depressants to enhance its sedative or euphoric effects (Hardon and Ihsan, 2014). The Drug Enforcement Agency (DEA) has reported cases of carisoprodol intoxication are increasing. Seizures induced by carisoprodol misuse increased from 3,988 in 2008 to more than 5000 in 2010, higher than other abused drugs including like lorazepam and methylphenidate (Witenko et al., 2014). Increasing popularity of carisoprodol among high school students is also a topic of concern. According to Monitoring the Future National Survey on Drug Use (2009), annual non-medical use of carisoprodol among all high school seniors was 1.3% and 1.4% in 2007 and 2008, respectively (Reeves et al., 2012). These numbers are higher than other prescription drugs that are abused like chlordiazepoxide (0.2%) and lorazepam (0.4%) and comparable to clonazepam (1.3%) (Reeves et al., 2012). Considering the danger posed by carisoprodol and its increasing abuse rate, an understanding

of its mechanism of action is of interest. Its physiological effects have generally been attributed to actions of its primary metabolite, meprobamate (Reeves et al., 1999b) on GABA type A receptors (GABA_ARs), the predominant inhibitory neurotransmitter receptor in the brain (Sigel and Steinmann, 2012). However, it is now known that carisoprodol can directly gate and allosterically modulate the GABA_AR, likely through a novel site(s) of action (Gonzalez et al., 2009b), and thus many actions may be attributable to the parent drug carisoprodol. In this overview, the current understanding with regard to the abuse potential of carisoprodol, therapeutic and abuse-related actions of this drug, and possible molecular actions that underlie these effects is discussed.

Clinical Use of Carisoprodol

Carisoprodol was first approved in 1959 by the Food and Drug Administration (FDA) for clinical use as a skeletal muscle relaxant (Fass, 2010). It is/has been marketed in the United States under the brand name Soma, Soridol and Rela in the United States (Littrell et al., 1993a), Carisoma in the United Kingdom, and under the names Sonoma, Somadril, Somacid, Scutamil C, Relacton-C, Mio Relax, and Relaxibys in other countries (Reeves et al., 2012). Carisoprodol is commonly prescribed for acute musculoskeletal spasm and associated pain in adults. The analgesic, muscle relaxant and sedative effects of carisoprodol are the basis for its use in the alleviation of lower back pain and in the short-term treatment of painful, acute musculoskeletal conditions (Toth and Urtis, 2004). Like other muscle relaxants, carisoprodol is often prescribed as an adjunct to rest or physical therapy and is also

available in preparations with other analgesics such as aspirin or codeine (Soma[®] Compound or Soma[®] Compound with codeine) (2004). The onset of action is rapid and effects last for 4-6 hours (Littrell et al., 1993a). It is available in 250 mg and 350 mg tablets, and is administered 3-4 times a day for a maximum duration of 2-3 weeks. Carisoprodol was the 2nd most prescribed muscle relaxant in the US in 2000, accounting for 21% of all skeletal muscle relaxant prescriptions. Together with cyclobenzaprine and metaxalone, these three drugs accounted for 45% of all skeletal muscle relaxant prescriptions dispensed in 2003 and 2004 (Luo et al., 2004; Toth and Urtis, 2004). A market intelligence corporation, IMS Health, reported 10.6 million prescriptions were dispensed in 2008 alone. The most common side effects of carisoprodol when taken medicinally are drowsiness, dizziness, and headaches (Bramness et al., 2007a). Other side effects include nausea, vomiting, hypotension, tachycardia, ataxia, vertigo, tremors and seizures. Adverse effects are generally observed after high dose administration or sudden cessation. Toxic doses of carisoprodol elicit agitation, myoclonus and bizarre movements (Bramness et al., 2005a; Roth et al., 1998).

Pharmacokinetics and Metabolism

Carisoprodol is a white crystalline powder with mild characteristic odor and a bitter taste. It is a racemic mixture of two stereoisomers, (*RS*)-2-[[[(aminocarbonyl)oxy]methyl]-2-methylpentyl isopropylcarbamate. Its molecular weight is 260.33 and its octanol\water partition coefficient is 2.36. Carisoprodol solubility is independent of pH. It is freely soluble in organic solvents DMSO, chloroform, acetone and alcohol and moderately soluble in water

(30 mg/100 mL at 25 °C (Littrell et al., 1993a). The structure of carisoprodol is closely related to meprobamate, differing only by a hydrogen atom substituting for the isopropyl group on one of the carbamyl nitrogens (Figure 1). Carisoprodol's structure is also closely related to felbamate, an anti-epileptic drug used to treat partial and generalized seizures. However, later reports of liver failure and aplastic anemia limits its usage to severe refractory seizures. The CYP2C19 genotype influences the pharmacokinetics of carisoprodol, and thus carisoprodol and meprobamate blood concentrations. EMs and IMs (extreme and intermediate metabolizers) metabolize carisoprodol with a half-life of 1.5 hours (Dalen et al., 1996; Olsen et al., 1994). Around 2-3% of Caucasians are PMs (poor metabolizers); they eliminate carisoprodol with a half-life of more than 6 hours and generate meprobamate to a small extent (Tamminga et al., 2001). Carriers of deficient CYP2C19 alleles accumulate higher concentrations of carisoprodol that may lead to intoxication, while potentially high levels of the active metabolite meprobamate can accumulate in CYP2C19*17 carriers. However, CYP2C19 genetics alone do not appear to play a significant role in carisoprodol-related mortality (Hoiseth et al., 2012). Instead, interaction with other drugs likely plays a key role in carisoprodol-related fatalities (McIntyre et al., 2012). Postmortem evaluation of carisoprodol concentration in blood and liver has shown carisoprodol concentrations as low as 15 mg/L in blood and 50mg/kg in liver can be fatal in the presence of other sedative/hypnotics like alcohol, diazepam, alprazolam and temazepam (McIntyre et al., 2012).

Age, sex and co-administration of other drugs that interact with CYP2C19 enzyme also affect carisoprodol metabolism and thus blood concentrations (Tse et al., 2014). A retrospective study conducted on 14,965 subjects assessed effects of age, sex and drugs that inhibit CYP2C19 enzymes (esomeprazole, fluoxetine or omeprazole). Metabolic rates were twice as high in young subjects compared to elderly, and were approximately 20% higher in females than males (Tse et al., 2014). Also, esomeprazole and fluoxetine, but not omeprazole, significantly decreased the metabolic rate of carisoprodol. Use of oral contraceptives inhibits the metabolism of carisoprodol in a CYP2C19 genotype-dependent manner; IMs using oral contraceptives accumulate more carisoprodol than EMs using them (Bramness et al., 2005b). As more than 25% of women aged 20-40 use oral contraceptives (Ronning, 2001), it is important for clinicians to be aware of the potential effects on carisoprodol concentration in those taking oral contraceptives.

Figure I-1. Metabolism of carisoprodol via cytochrome CYP2C19 enzyme. Carisoprodol is metabolized by cytochrome CYP2C19 enzyme via N-dealkylation of its isopropyl group to form active metabolite meprobamate. Carisoprodol also metabolized to an inactive metabolite, hydroxyl- carisoprodol, but to a very small extent by an unknown enzyme. Meprobamate and hydroxyl- carisoprodol are further metabolized to hydroxyl-meprobamate and conjugate and excreted by in urine.

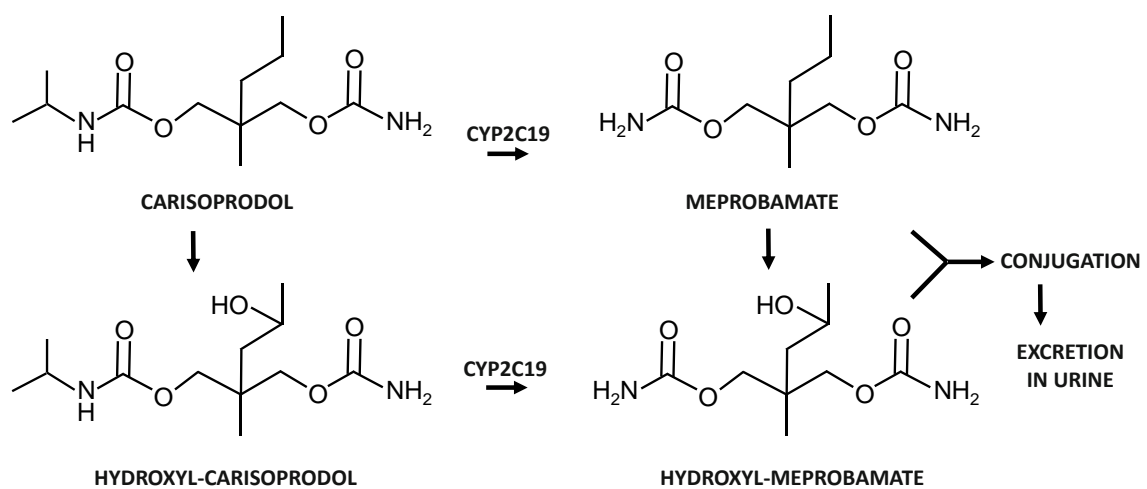


Figure I-1

Abuse and Withdrawal Syndrome

Carisoprodol was developed as an analog of meprobamate, promoted as having less abuse liability and better muscle-relaxing properties (Nebhinani et al., 2013). An early report suggested carisoprodol did not substitute for morphine or barbiturate and did not produce morphine- or barbiturate-like intoxication or withdrawal symptoms, and it was thus concluded carisoprodol does not pose abuse liability (Littrell et al., 1993a). However, more recent reports confirmed its abuse potential as well as subjective and psychological impairment and severe withdrawal syndrome that may predispose to seizures and even death (Fass, 2010; Gonzalez et al., 2009a; McIntyre et al., 2012; Ni et al., 2007; Reeves and Burke, 2010; Reeves et al., 2012; Zacny et al., 2012). The American Association of Poison Control Centers reported 2632 cases of intentional carisoprodol ingestion requiring medical attention in 2008 (Bronstein et al., 2009).

Studies conducted in healthy individuals showed a single supra-therapeutic 700 mg dose of carisoprodol produced both subjective and psychomotor impairments, whereas a single therapeutic dose (350 mg) produced mainly psychomotor impairments and minimum subjective impairments (Zacny et al., 2011). Administration of 10 mg oxycodone within 1 hour of 350 mg carisoprodol further affected the psychomotor impairments (Zacny et al., 2012). A study conducted in northern India also assessed dose-dependent effects of carisoprodol (Nebhinani et al., 2013). In that study, consumption of 1-3 tablets produced feelings of well-being and an energetic state, consumption of 4–10 tablets produced psychomotor excitement, cheerfulness, increased socialization and self-confidence, while

consumption of a one-time dose of 10 tablets produced an acute organic brain syndrome-like state with disorientation and partial amnesia of the events occurring during intake. In a study conducted by Reeves and Burke (Reeves and Burke, 2010), subjects consuming 12-30 tablets a day alone or in combination with other drugs like tramadol, benzodiazepines or alcohol reported euphoria and hallucinations. Misuse of carisoprodol has also been associated with suicide attempts (Bailey and Briggs, 2002).

In patients taking prescribed carisoprodol for extended use, the desire to continue its use is strong. Owens et al (Owens et al., 2007) found in approximately 80% of reported cases, patients continued to purchase carisoprodol even after their insurance declined to cover the bills. A survey of 40 individuals who had been taking carisoprodol for more than 3 months showed half of them had a history of drug abuse. Out of those 20 people, 40% admitted to taking more than the prescribed dose, 30% admitted taking for effects other than low back pain, 10% admitted taking to stimulate effects of other drugs being abused, and 5% admitted taking to counter the effects of other drugs (Reeves et al., 1999b). Collectively, the evidence suggesting carisoprodol has addictive properties and abuse potential is high. Of concern is the fact that physician awareness of the potential dangers of carisoprodol may be inadequate. Whereas 95 out of 100 physicians reported knowing meprobamate is a controlled substance, only 18 were aware that carisoprodol is metabolized to meprobamate (Reeves et al., 1999b). Now that carisoprodol is also scheduled, the recognition of dangers associated with its misuse should be improved.

Carisoprodol is passed to nursing infants during breastfeeding. Serum concentrations of carisoprodol and meprobamate were measured from a nursing mother who took 3 times the normal dose of carisoprodol per day for severe back spasms before and after uncomplicated delivery, and during the first month of breast feeding (Briggs et al., 2008). Significant amounts of carisoprodol and meprobamate were found in both the breast milk and the infant's serum, but no developmental toxicity was observed. Mild sedation in the baby while breast feeding was reported, however no signs of withdrawal were observed after feeding was stopped.

Carisoprodol abuse has increased rapidly in recent years. Low cost and easy accessibility compared to other illegal drugs make it an ideal choice for substance abusers. Abusers often combine carisoprodol with other psychoactive drugs to augment or alter their effects. For example, it may be combined with alcohol or benzodiazepines to increase their sedative effects, with cocaine to attenuate jitteriness associated with its use, and with other drugs to get synergistic relaxation and euphoria (Reeves and Burke, 2010; Reeves and Liberto, 2001). Individuals may substitute carisoprodol for opiates or benzodiazepines if these drugs are not accessible (Reeves et al., 1999a). As per the National Survey on Drug Use and Health in 2009, an estimated 2.9 million people in the United States admitted lifetime consumption of carisoprodol for non-medical purposes. In 2008, the National Forensic Laboratory information system reported carisoprodol as 1 of the 25 most frequently abused drugs (Fass, 2010). In 1996, the DEA proposed scheduling of carisoprodol, but the FDA and drug advisory committee did not take this action, feeling the evidence regarding

abuse potential of carisoprodol was insufficient (Fass, 2010). Following additional hearings on the possible scheduling of carisoprodol in 2010, carisoprodol was placed in schedule IV of the Controlled Substance Act of 1970 effective January 11, 2012 (see ref: Schedule of control substances). Since the earlier review, a better understanding of the potential mechanism underlying its actions had been developed, and abuse of carisoprodol had risen significantly. For example, in 2009, out of roughly 50,000 emergency department visits related to abuse of musculoskeletal relaxants, 30,000 visits were due to misuse of carisoprodol; this is double the number of visits related to carisoprodol abuse reported just five years earlier (Reeves et al., 2012). Considering the subjective, psychomotor impairment and CNS depressant effects of carisoprodol, it is not surprising that carisoprodol use increases risk of traffic accidents and accidents while operating heavy machine (Zacny et al., 2011). Indeed, carisoprodol and meprobamate have been ranked 7th out of the top 10 classes of drugs associated with driving under the influence of drugs, and this activity may in fact result in a charge of driving while intoxicated (Bramness et al., 2007b).

Carisoprodol abuse is not only an issue in the United States; its abuse has been documented in many other countries (Bramness et al., 2007a; Bramness et al., 2007b; Hardon and Ihsan, 2014; Nebhinani et al., 2013). In 2007, Norway scheduled Carisoprodol as class “A” drug, which is the highest scheduling level in Norway and a category that includes narcotics and hypnotics (Hoiseth et al., 2009). Recently, the Committee for Medicinal Products for Human Use concluded the abuse potential associated with carisoprodol outweighs its benefits as a therapeutic drug; carisoprodol was removed from the market by

the European Medicines Agency in 2008 and is only available to those for whom there are no other alternatives (Hoiseth et al., 2009). In South Sulawesi, sex workers spend nearly all their earnings to buy carisoprodol (Hardon and Ihsan, 2014). They take up to 10 pills a day, often along with alcohol and other psychoactive prescription drugs, to make their work palatable. Carisoprodol has become a cross-border problem as well. Several thousands of pills can easily be purchased at a time in Mexico to be smuggled over to the United States and sold to teenagers (Davis, 2009). Correspondingly, the Los Angeles police department reported carisoprodol is one of the prescribed drugs they encounter most frequently at the US-Mexico border crossing (Reeves and Burke, 2010).

A significant problem with carisoprodol is that tolerance to its effects develops rapidly, and increased consumption of tablets often follows. Subsequent abrupt cessation results in withdrawal syndrome (Gatch et al., 2012). Abrupt cessation of carisoprodol administration in humans (100 mg/kg/day, 5 times the normal daily dosage) results in insomnia, abdominal cramps, headache, chills and nausea (46). Hallucinations, delusions, tremors, seizures and even death have also been reported (Ni et al., 2007; Reeves et al., 2004; Reeves and Burke, 2010). Prisoners in Norway who had been taking 700 - 2100 mg/day of carisoprodol for at least 9 months showed withdrawal symptoms including insomnia, anxiety, irritability and muscular pain upon sudden discontinuation of drug intake (Wyller et al., 1991). Whereas withdrawal symptoms are generally associated with cessation of large doses of carisoprodol, they have been observed with cessation of as few as 4 to 8 tablets of carisoprodol per day (Reeves et al., 1997). Whereas toxic and withdrawal symptoms have a

notable GABAergic component (below), other systems may be affected. Bramness et al (Bramness et al., 2005a) reported 4 cases where subject had consumed up to 65 pills of carisoprodol alone or along with other sedative/hypnotics; these subjects showed characteristic effects that might be associated with serotonergic activation, such as tachycardia, hypertension, involuntary, robot-like, choreiform movements, tremors and shivering (Bramness et al., 2004; Roth et al., 1998). In that study, however, signs and symptoms included in the classification of serotonergic syndrome were nonspecific; because a complete drug screening was not performed, one cannot rule out the possibility that other drugs present were eliciting the serotonergic effects. Currently, a suitable and efficient treatment for carisoprodol intoxication and withdrawal is not available. Withdrawal syndromes are treated with barbiturates and benzodiazepines to suppress anxiety and agitation (Reeves et al., 2007). Treating carisoprodol overdose is more complicated because of the presence of serotonergic-like symptoms noted above. A benzodiazepine antagonist is generally given in cases of carisoprodol intoxication, but considering carisoprodol does not act at the benzodiazepine site (below), this is not an entirely appropriate approach.

Pharmacology of Carisoprodol

Carisoprodol mediates its effects centrally rather than through direct skeletal muscle relaxation (Gonzalez et al., 2009b). Inhibition of inter-neuronal transmission in the descending reticular formation and spinal cord is one of the mechanisms proposed for its muscle relaxant properties. Whereas the precise mechanism of action of carisoprodol is not

fully understood, it has been thought that carisoprodol exerts its effects via its metabolite, meprobamate. Meprobamate is a sedative-hypnotic that was commonly used in the treatment of anxiety. It is currently classified as a schedule IV controlled substance at the federal level, with abuse potential comparable to that of benzodiazepines (Mounier et al., 2012; Roache and Griffiths, 1987). Meprobamate was introduced in 1955 under the brand names Miltown and Equnil, but within a few years was replaced by benzodiazepines because of high abuse liability (Reeves et al., 2012). Although the central mechanism of action of meprobamate has also not been fully elucidated, GABA_A receptors are a key target. Meprobamate allosterically potentiates GABA_A receptors and directly gates the receptor at millimolar concentrations (Rho et al., 1997). Meprobamate intoxication causes cardiogenic hypotension, CNS depression, flaccid muscles and loss of tendon reflexes (Allen et al., 1977). As noted, it was assumed therapeutic effects and abuse potential of carisoprodol are due to its metabolism to meprobamate (Littrell et al., 1993b). Following a single 700 mg dose of carisoprodol, serum levels of meprobamate surpass those of carisoprodol in 2.5 hours, and approximately 90% of carisoprodol is metabolized to meprobamate in 6 hours (Olsen et al., 1994). Despite the likely contribution of meprobamate to the therapeutic and adverse effects of carisoprodol, the pharmacological and physiological effects of carisoprodol are somewhat distinct, suggesting carisoprodol has its own actions (Gonzalez et al., 2009a). For example, supratherapeutic doses of meprobamate mainly result in CNS depressant effects such as dizziness, drowsiness, ataxia, tremors, blurred vision and headache, whereas agitation and bizarre movements occur with toxic levels of carisoprodol.

Moreover, signs of toxicity are observed within 30 minutes of overdose of carisoprodol ($t_{1/2}$ 1.5 hours), before it is metabolized to meprobamate ($t_{1/2}$ 11 hours) (Littrell et al., 1993a).

Gonzalez et al., using whole cell patch electrophysiology in HEK cells, showed carisoprodol itself allosterically modulates and directly activates GABA_ARs (GABA type A receptors) (Gonzalez et al., 2009b). Carisoprodol inhibits the receptor at high concentration, eliciting rebound currents upon termination of drug application, as observed with barbiturates. In vivo studies showed maximal depression of motor activity was observed within 10 minutes of carisoprodol administration, a timeframe inconsistent with effects being due to conversion to meprobamate (Gonzalez et al., 2009b). In addition, in rats trained to discriminate carisoprodol from saline, the GABAergic ligands pentobarbital, chlordiazepoxide and meprobamate substituted for carisoprodol. These results suggest GABA_A receptors as potential targets of carisoprodol, and demonstrate its capacity to enhance the sedative effects of CNS depressants, contributing to its potential for abuse. Discriminative stimulus effects of carisoprodol were blocked by the barbiturate antagonist bemegride, but not by the benzodiazepine antagonist flumazenil. Similarly, carisoprodol-gated currents were blocked by bemegride but not by flumazenil. These results rule out the involvement of the benzodiazepine binding site in carisoprodol modulation of GABA_A receptors. Recently, Gatch et al. (Gatch et al., 2012) characterized tolerance and dependence potential in mice. Tolerance (measured as loss of righting reflex) developed quickly, as mice showed a decrease in the loss of righting reflex following just 4 doses of carisoprodol. Whereas spontaneous withdrawal symptoms were not produced within 24 hours following

withdrawal from carisoprodol, precipitated withdrawal signs were observed with administration of either the benzodiazepine antagonist flumazenil or the barbiturate antagonist bemegride. Although drug discrimination and electrophysiological studies suggest possible overlap of the mechanism of action of carisoprodol and barbiturate effects, site-directed mutagenesis on homomeric $\rho 1$ GABA receptors have shown functional domains are not equivalent. Specifically, mutation of tryptophan to methionine at position 328 of the $\rho 1$ subunit ($\rho 1W328M$) confers pentobarbital direct gating sensitivity to insensitive wild type $\rho 1$ GABA receptors, but this mutation does not confer sensitivity to carisoprodol (Gonzalez et al., 2009b). Thus whereas carisoprodol shares several properties with barbiturates and a barbiturate antagonist can attenuate some of its effects, the two molecules interact at distinct sites on the GABA_A receptors.

GABA_A Receptors

GABA is the major inhibitory neurotransmitter in the vertebrate central nervous system and in the central and peripheral nervous system of invertebrates (DeLorey and Olsen, 1992). GABA released from GABAergic neurons generate postsynaptic neuronal inhibition through a variety of membrane bound receptors, GABA_(A-C) type receptors (Sigel and Steinmann, 2012). These GABA receptors can be divided into two categories, ligand-gated ion channels (GABA_A and GABA_C receptors) and metabotropic receptors (GABA_B receptor) that are G-protein coupled receptors (Bormann, 2000). GABA_A receptors are the most predominant inhibitory receptor in human CNS and are selective for Cl⁻ ions (DeLorey and

Olsen, 1992). Influx of chloride ions hyperpolarize the post-synaptic neurons and is essential for the balance between the neuronal excitatory and inhibitory signaling that is critical for normal brain function (McBain et al., 2015; Moss and Smart, 2001). Excessive inhibition or lowered excitation can lead to sedation, hypnosis, sleep, depression and coma whereas excessive excitation or lowered inhibition can result in anxiety, convulsions, restlessness and insomnia (Johnston, 2005). Precise symptoms depend upon the exact region of the brain and nerve cells that are out of balance (Whiting, 2003). Any extreme imbalance can lead to death. The aim of the therapeutic drugs that target GABA_AR is to restore balance between inhibition and excitation (Rudolph and Mohler, 2006; Rudolph and Mohler, 2014).

Structure of GABA_A receptors

GABA_A receptors are a member of the cys-loop receptor superfamily, a structural feature shared by entire superfamily (13 amino acids loop contained by a disulphide bond in the extracellular N-terminal) (Corringer et al., 2012; daCosta and Baenziger, 2013). Other members of the superfamily include nicotinic acetylcholine, type 3 5-hydroxytryptamine, GABA_C and glycine receptors. nACh and 5HT₃ receptors are selective for cations that depolarize (excite) the neurons whereas GABA_A, GABA_C and glycine receptors are selective for anions that hyperpolarize (inhibit) the neurons (Miller and Smart, 2010). GABA_AR are heteromeric assemblies of five subunits forming a central ion conducting pore (figure I-2) (Corringer et al., 2012). Multiple GABA_AR subunits as well as their isoforms have been

identified including $\alpha(1-6)$, $\beta(1-3)$, $\gamma(1-3)$, ρ , δ , ε and θ (Barnard et al., 1998; Olsen and Sieghart, 2008).

Figure I-2. Molecular homology model of GABA_A receptor. **A**, Side view of pentameric GABA_A receptor representing five subunits, dimensions and position of the various domains of GABA_A receptor on the cell membrane; α subunit in red, β subunit in blue and γ subunit in yellow. **B**, Top view of pentameric GABA_A receptor representing channel pore and its dimensions from top of the channel pore to channel gate. Created in Dr. Peter Gannett's lab with the help of Dr. Robyn Ayscue. Template was made available online by Dr. Bergmann (Bergmann et al., 2013).

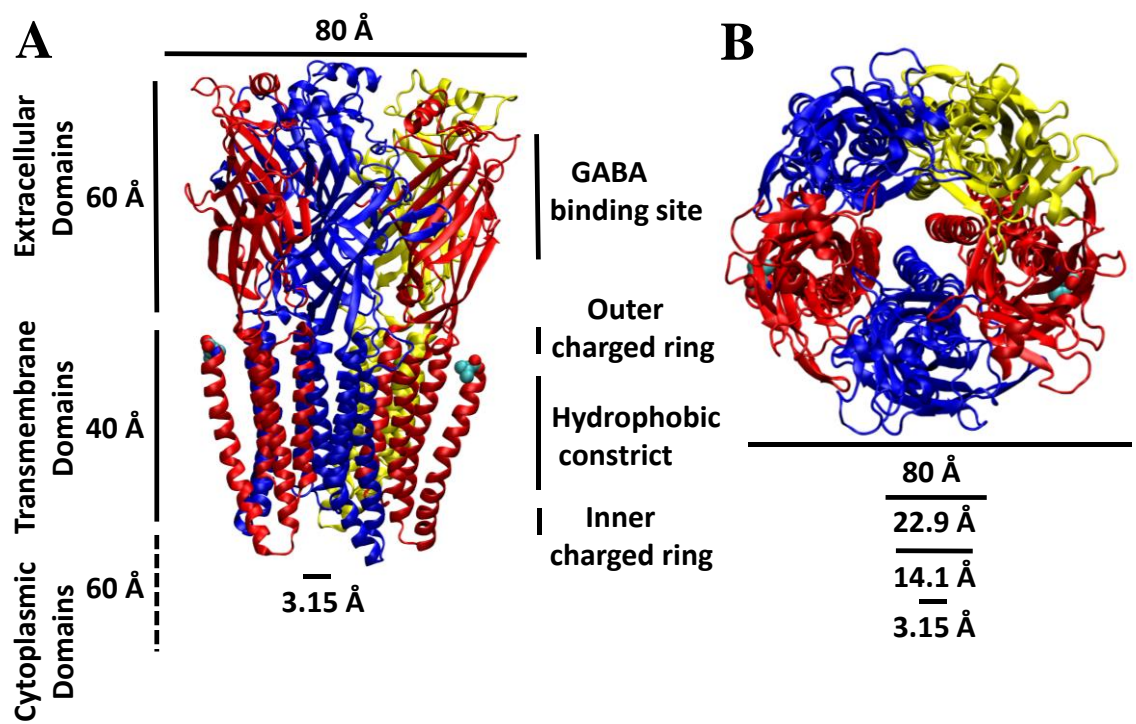
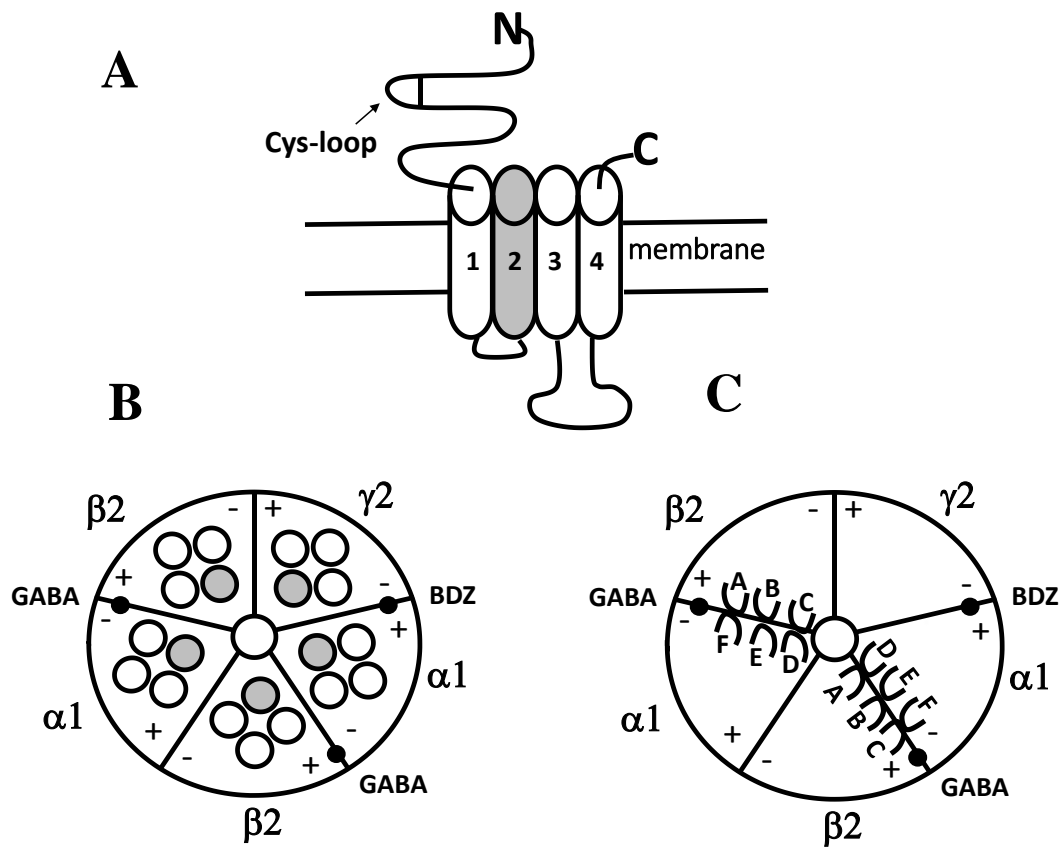


Figure I-2

Each subunit of the GABA_AR is composed of a large extracellular N terminus, four transmembrane helices (TM1–TM4), where TM2 lines the ion channel pore, one extracellular TM2–TM3 loop, two intracellular loops (TM1–TM2 and TM3–TM4), and an extracellular C terminus (figure I-3). Considering the diverse number of subunit isoforms, a large number of GABA_AR can be assembled. However, it has been verified that $\alpha\beta\gamma$ and $\alpha\beta\delta$ receptor (2:2:1 stoichiometry) isoforms are the predominant GABA_ARs in the brain (Quirk et al., 1994) with $\alpha\beta\gamma$ GABA_ARs mainly located within the synapses and $\alpha\beta\delta$ receptors are preferentially expressed at extra- or perisynaptic membranes (figure I-4) (Belelli et al., 2009). While synaptic GABA_A receptors exhibit phasic inhibition, extra-synaptic GABA_A receptors exhibit tonic inhibition, persistently activated by low ambient GABA levels present in the extra-cellular space spilled from synaptic region (Farrant and Nusser, 2005; Glykys and Mody, 2007; Herd et al., 2009). Extra-synaptic GABA_A receptors have low efficacy but high affinity for GABA compared to other ligands such that GABA is considered partial agonist at these receptors (Bianchi and Macdonald, 2003). Single channel studies have shown $\alpha\beta\delta$ receptors display brief openings whereas $\alpha\beta\gamma$ receptors exhibit burst openings with longer open duration (Akk et al., 2004; Feng et al., 2004; Wohlfarth et al., 2002). Synaptic receptors that respond to millimolar concentrations of GABA exhibit fast inhibitory postsynaptic potentials (IPSPs), whereas extra-synaptic receptors which respond to micromolar concentrations of GABA spilled in the extra-cellular space consistently exhibit slower IPSPs (Belelli et al., 2009).

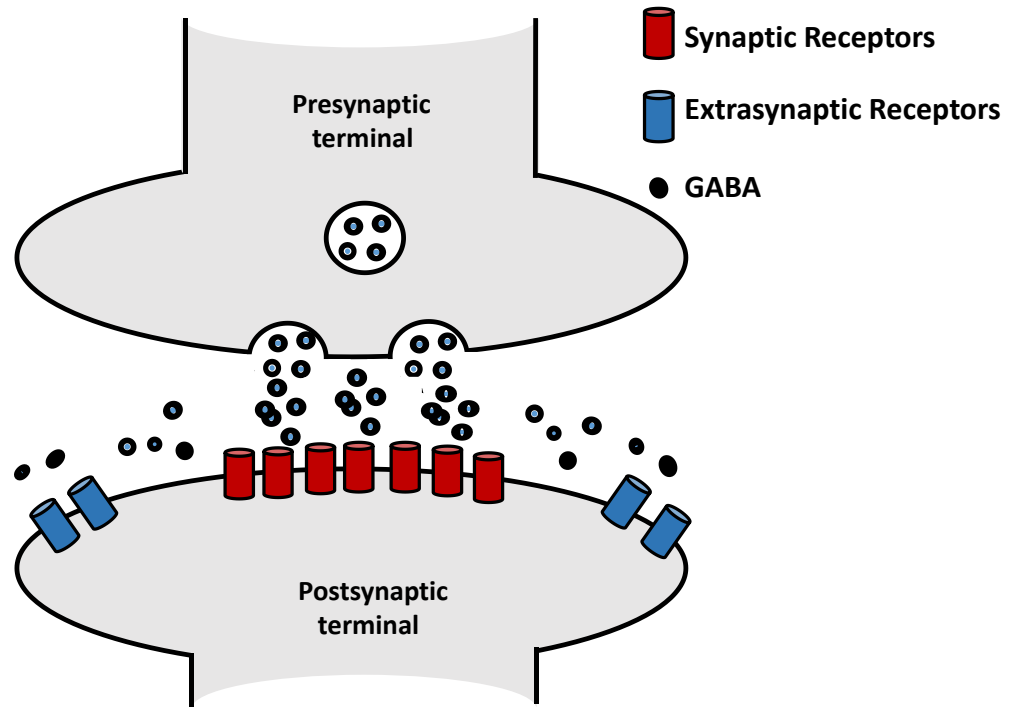
Figure I-3. Schematic representation of major GABA_A receptor isoform and topological model of a single subunit. **A**, Side view of a single subunit of GABA_A receptor representing a large extracellular N terminus showing cys-loop, four transmembrane helices (TM1–TM4), where TM2 lines the ion channel pore (grey), one extracellular TM2–TM3 loop, two intracellular loops (TM1–TM2 and TM3–TM4), and an extracellular C terminus. **B**, Top view of GABA_A receptor representing position of five subunits in anti-clockwise ($\beta 2:\alpha 1:\beta 2:\alpha 1:\gamma 2$). Positive side of the $\beta 2$ subunit face negative side of the $\alpha 1$ subunit and negative side of $\alpha 1$ subunit faces positive side of $\gamma 2$ subunit. The Transmembrane2 (TM2) (grey) of each subunit line the channel pore. GABA bind at the interface of $\alpha 1$ and $\beta 2$ subunits and benzodiazepines (BDZ) at the interface of $\alpha 1$ and $\gamma 2$ subunit. **C**, Representation of binding pocket loops of GABA on GABA_A receptors. Principal side on β^+ subunit; loop A, B and C and complementary side on α^- subunit; loops D, E and F.



Subunit Diversity: α_{1-6} , β_{1-3} , γ_{1-3} , δ_1 , θ_1 , ε_1

Figure I-3

Figure I-4. Localization of synaptic and extrasynaptic GABA_A receptors on postsynaptic neurons and their properties.



Synaptic GABA_A Receptors



- GABA released at synapse
- Phasic Inhibition
- GABA: Full agonist
- $\alpha\beta\gamma$ configuration receptors

Extrasynaptic GABA_A Receptors



- GABA spilled from synapse
- Tonic Inhibition
- GABA: Partial agonist
- $\alpha\beta\delta$ configuration receptors

Figure I-4

Ion conduction and mechanism of action

GABA_ARs are selective for Cl⁻ ion with some permeability for bicarbonate anions (Kaila et al., 1989). However, if the extracellular concentration of Cl⁻ decreases due to down regulation of KCC2 Cl⁻ ion transporter, opening of GABA_AR channel pore results in efflux of Cl⁻ ions leading to depolarization of the neurons and thus, excitation (Coull et al., 2005). Equilibrium or reversal potential of Cl⁻ ion is close to the resting membrane potential and thus, GABA_ARs have the capacity to exhibit a different form of dynamics where a small change in Cl⁻ ion concentration can lead to significant changes in the nature of GABA_AR-mediated transmission (Wright et al. 2011). For example, if equilibrium potential of Cl⁻ is more negative than the resting membrane potential GABA_AR activation will result in membrane hyperpolarization and inhibition. If equilibrium potential of Cl⁻ is more positive than the resting membrane potential stimulating GABA_ARs activation will result in membrane depolarization and thus excitation. Excitatory GABA_A receptors are well documented in early development and neuropathic pain because of low extracellular Cl⁻ levels (Coull et al., 2005; Wright et al., 2011).

Binding of a neurotransmitter “GABA” on GABA_AR initiates a series of molecular motions (conformational wave) that begin in the binding pocket, followed by movement in pre TM1 regions to TM(1-4) helices to open the ion pore, a process called coupling (Miller and Smart, 2010). Binding of the GABA at the binding pocket or site causes the channel to adopt a conformational open stable state. GABA_A receptors have two binding pockets for GABA at the interface of α and β subunits in the extracellular domains region. The binding

pocket is formed by three amino acid loops (A, B and C) on β^+ subunit “principal side” and three amino acid loops (D, E and F) on α^- subunit “complementary side” (figure I-3C)(Ernst et al., 2003). GABA_A receptor extracellular domains also have binding pocket for benzodiazepines at the interface of α and γ subunits (Ernst et al., 2005). How binding of a ligand leads to channel opening is not clear yet and many theories have been proposed and studied. One of the regions suggested to be involved in channel gating is a short extracellular loop between TM2-TM3 (also called TM2-3 linker) (Thompson et al., 2010). Movement in TM2-3 linker mediated by extracellular domains, destabilizes the hydrophobic ring in the channel which displaces from the center of the pore to the space between the inner and outer rings, ultimately opening the channel (Miyazawa et al., 2003). Also, inherited mutations in this region have been documented which alters the channel gating without affecting ligand binding (Campos-Caro et al., 1996; Kusama et al., 1994).

Interestingly, opening of cys-loop receptors or GABA_A receptors can occur in the absence of ligand or GABA as well, but at a very low frequency (Thompson et al., 2010). The binding of ligand increases the probability of channel opening and maximizes as the number of bound ligands reaches two. Since rate of channel opening can be quicker than the dissociation of the ligand, multiple opening states can occur during a single ligand binding (Burzomato et al., 2004). How efficiently ligand docking at binding pocket initiates channel opening and stabilize the open states is dependent upon how efficient is the communication or conformation wave between the extracellular domains to transmembrane domains. Different ligands have different efficacies on GABA_A receptors with varying open probability.

Full agonists initiate channel opening faster or stabilize the channel opening for a longer duration compared to partial agonists (Baumann et al., 2003).

Modulation of GABA_A receptors

Like other proteins, GABA_A receptors are regulated by post-translational modification. Protein kinases have been identified that phosphorylate specific amino acids residues of specific GABA_A receptor subunits and modulate channel gating, surface stability and trafficking (Adams et al., 2015; Huson et al., 2007). Apart from this covalent modification, GABA_A receptors are modulated by endogenous and exogenous molecules and may potentiate or suppress the natural response of GABA (Johnston, 2005). Considering the importance of GABA_A receptors in the central nervous system, several classes of exogenous drug molecules are used. Examples include benzodiazepines, barbiturates, general anesthetics, ethanol, the competitive antagonist, biculline and the channel blocker, picrotoxin (Olsen, 2014). These molecules increase GABA-mediated synaptic inhibition either by directly activating GABA_A receptors (Barbiturates) or, by potentiating the action of GABA on GABA_A receptors (Barbiturates/ Benzodiazepines). This latter action is called positive modulation and is believed to be involved in molecules acting on allosteric sites on GABA_A receptors remote from the GABA recognition sites (orthosteric sites) (figure I-5). Such allosteric sites are regarded as good targets for the development of subtype specific drugs since there is generally greater diversity between receptor subtypes in amino acid sequence at allosteric sites than at orthosteric sites (Johnston, 1996). Molecules that reduce the action of

GABA on GABA_A receptors are known as negative allosteric modulators (Biculline and Picrotoxin). Molecules that block the actions of both positive and negative allosteric modulators are known as neutralizing allosteric modulators (benzodiazepine ‘antagonist’ flumazenil)(Christopoulos, 2002). Neurosteroids (Allopregnanolone and THDOC) are the most potent positive endogenous modulators of GABA_ARs (Akk et al., 2009). Neurosteroids have been found to be involved in various disorders, like depression, anxiety, alcohol abuse, epilepsy, and neurodegenerative diseases (MacKenzie and Maguire, 2013). It has been shown that the endocannabinoid (2-arachidonoylglycerol) specifically positively modulates $\beta 2$ subunit-containing GABA_A receptors through a binding site located in TM4 (Ernst et al., 2005). However, physiological role of this modulation is still not clear.

It is suggested that GABA_A receptors are modulated by a large range of molecules because of the numerous binding cavities present in the transmembrane region of the receptor for these molecules (Ernst et al., 2005). Besides the GABA-binding site, GABA_ARs have binding sites for several clinically important drugs, including anxiolytics, sedative-hypnotics, muscle relaxants, and anesthetics (Johnston, 1996). GABA binding site is located at the interface of the $\alpha 1$ and $\beta 2$ subunits in the extracellular domains, whereas the pentobarbital/general anesthetics (propofol) binding sites are believed to be positioned in the water accessible region located between the four TM helices of the receptor (Li et al., 2006). Moreover, the general anesthetics propofol and etomidate also allosterically modulate and directly gate GABA_ARs through single site of action (Bali and Akabas, 2004) whereas distinct GABA_AR sites confer these properties to neurosteroids (Hosie et al., 2006). Subunit

composition and arrangement determine drug selectivity (Dawson et al., 2005; Rudolph and Knoflach, 2011). Critical questions are whether the allosteric and direct gating effects of carisoprodol are mediated via a single or multiple sites and how does it modulate GABA_A receptors.

Figure I-5. Representation of chloride currents gated by GABA_A receptor ligands and their pharmacological properties. **A**, Representative traces of GABA_AR ligands having only positive allosteric modulation effect and not direct gating action of their own. **B**, Representative traces of GABA_AR ligands having both positive allosteric modulation effect and direct gating action. **C**, Representative traces of GABA_AR ligands having only negative allosteric modulation effect and not direct gating action of their own.

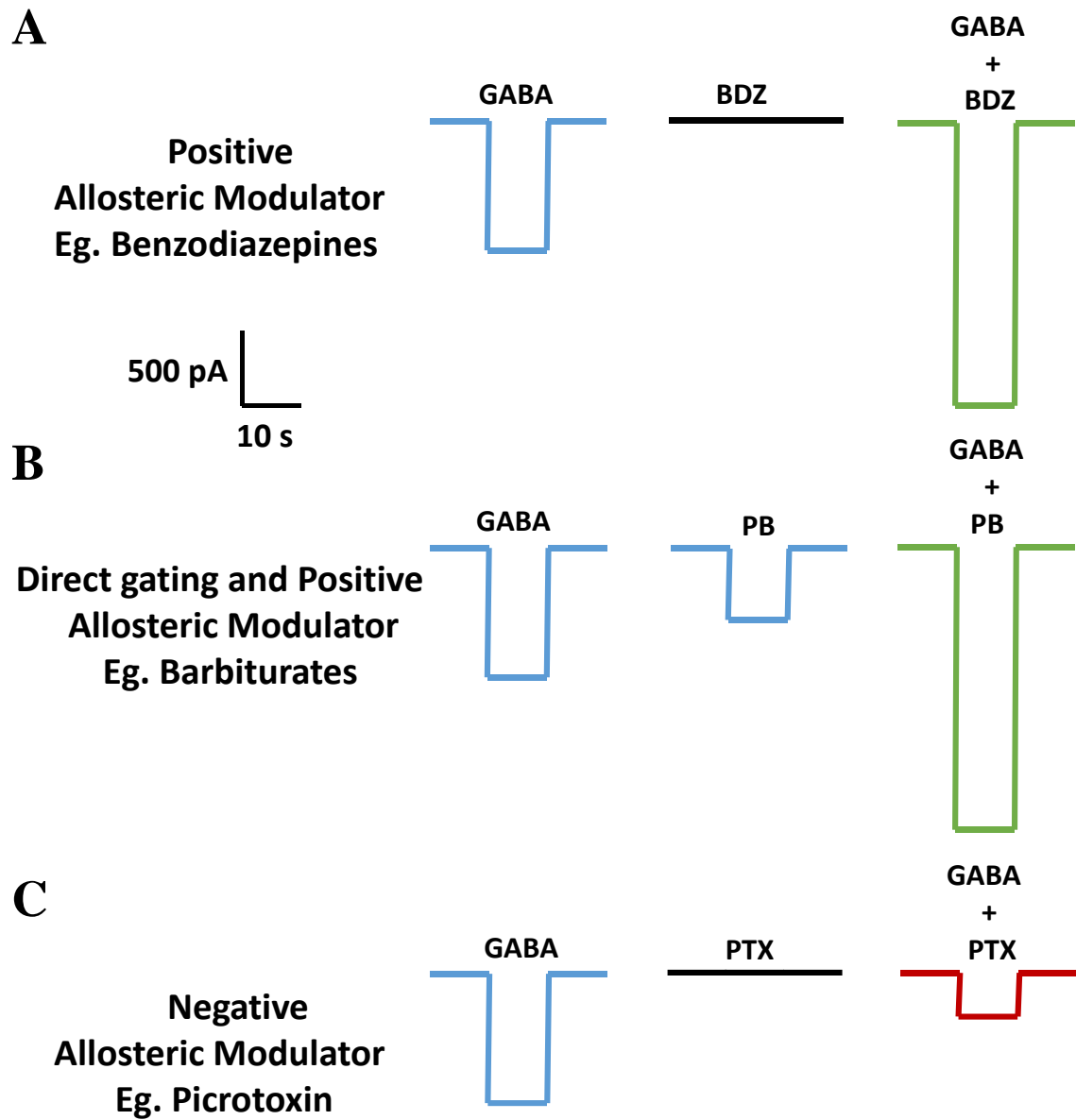


Figure I-5

Distribution and Physiological or Pharmacological Role of GABA_AR Subunits

Approximately 20-30% of the neurons in the CNS are GABAergic (Bormann, 2000). Despite diversity of GABA_A receptor subunit isoforms, approximately 60% of all GABA_A receptors consist of $\alpha 1\beta 2\gamma 2$ configuration and 90% of GABA_A receptors contain $\gamma 2$ subunit (Fritschy and Mohler, 1995). Approximately 15-20% of GABA_A receptors have subunit combination of $\alpha 2\beta 3\gamma 2$, approximately 10-15% have the $\alpha 3\beta x\gamma 2$ configurations, and approximately 5% have the $\alpha 4\beta x\gamma$ or $\alpha 4\beta x\delta$ configurations, less than 5% have the $\alpha 5\beta x\gamma 2$ configurations and similarly less than 5% have the configuration of $\alpha 6\beta n\gamma 2$ subunit combinations (Table 2). It is interesting to note, GABA_A receptors with two different α subunits and without γ or δ subunit have also been verified to express (Benke et al., 2004).

Given that GABA is the major inhibitory neurotransmitter, it is not surprising that GABA is involved, directly or indirectly, in many disorders of brain function (MacKenzie and Maguire, 2013; McBain et al., 2015). The major disorders for which GABA_A receptors represent major therapeutic targets include anxiety disorders, cognitive disorders, epilepsies, mood disorders, schizophrenia and sleep disorders (Johnston, 1996; Rudolph and Mohler, 2006; Rudolph and Mohler, 2014). Functional or physiological role of each GABA_A receptor depends upon both subunit composition and localization of the receptor (Dawson et al., 2005). Malfunctioning of tonic inhibition via δ expressing extra-synaptic GABA_A receptors has been documented to be involved in neurological disorders like depression (Maguire et al., 2005), schizophrenia (Maldonado-Aviles et al., 2009) and some forms of epilepsy (Cope et al., 2009) and thus, extra-synaptic receptors are proving to be target of emerging clinical drugs

(Brickley and Mody, 2012). Also, potentiation of tonic inhibition by neurosteroids, general anesthetics, anti-epileptics and sedatives/hypnotics is well verified (Bianchi and Macdonald, 2003; Feng et al., 2004).

An understanding of subunit isoform association with physiologic effects of GABAergic signaling has also developed over the past several years (Olsen and Sieghart, 2009). In an early study using knock-in technology to study roles of a specific amino acid in the $\alpha 1$ receptor (Histidine at position 101) (Rowlett et al., 2005; Rudolph et al., 1999), mutant mice expressing $\alpha 1$ (H101R) GABA_A receptors showed no sedative and anterograde amnesic effects of diazepam, and anticonvulsant effects were significantly reduced. Equivalent knock-in mutations of the conserved histidine were subsequently studied in $\alpha 2$ and $\alpha 3$ receptors (Crestani et al., 2001). In $\alpha 2$ (H101R) mice, anxiolytic and myorelaxant effects of diazepam were completely abolished, while sedative effects were present. In $\alpha 3$ (H126R) mutant mice, myorelaxant properties of diazepam were reduced (present at high diazepam doses) while sedative and anxiolytic properties were intact. These results demonstrated diazepam mediates its muscle relaxant effects mainly through $\alpha 2$ containing GABA_A receptors, and also through $\alpha 3$ GABA_A receptors at high concentrations. A drug selective primarily for $\alpha 2$ -expressing GABA_A receptors would likely have better muscle relaxant properties, less abuse potential and fewer sedative effects (Rudolph and Knoflach, 2011)

Table I-1. Localization of GABA_A receptor subtypes and their physiological and pharmacological role. Adapted from (Rudolph and Mohler, 2014).

α subunit Isoform	Identified Configurations	Pharmacological and Physiological Role	Synaptic or Extrasynaptic
$\alpha 1$ 60% of all GABA _A Rs	$\alpha 1\beta 2\gamma 2$	Sedation, Amnesia, Anticonvulsant action, Dependence and Addiction.	Synaptic
$\alpha 2$ (15-20)%	$\alpha 2\beta 3\gamma 2$	Anxiolysis, Antihyperanalgesia, Muscle relaxation and Cognitive impairment	Synaptic
$\alpha 3$ (10-15)%	$\alpha 3\beta 2,3\gamma 2$	Anxiolysis, Muscle relaxation, Sensorimotor gating and Antihyperanalgesia	Synaptic
$\alpha 4$ less than 5%	$\alpha 4\beta 2,3\delta$	Memory, Anxiety, Nociception, Sleep and Neuropsychiatric disorder	Extrasynaptic
$\alpha 5$ less than 5%	$\alpha 5\beta 3\gamma 2$	Sensorimotor gating, Cognitive impairment, Muscle relaxation, Autism, Down Syndrome and Schizophrenia	Synaptic and Extrasynaptic
$\alpha 6$ less than 5%	$\alpha 6\beta 2,3\gamma 2$; $\alpha 6\beta 2,3\delta$	Memory, Anxiety, Nociception, Sleep disorder, abdominal obesity and cortisol secretion	Synaptic and Extrasynaptic

Table I-1

Mechanism of Addiction of Benzodiazepines

The mesolimbic system is well documented for its involvement in the mechanism of addiction of opioids, ethanol, psychostimulants and nicotine (Luscher and Ungless, 2006). These drugs increase dopaminergic transmission in the mesolimbic system, increasing the levels of dopamine in the nucleus accumbens. With regard to additive property at the GABA_A receptors, the ligand for which we have the most knowledge are the benzodiazepines. Tan et al. showed $\alpha 1$ -containing GABA_A receptors are involved in addictive properties of benzodiazepines, and their activation elicits an increase in nucleus accumbens dopamine levels (Figure I-6) (Tan et al., 2010). GABAergic neurons in ventral tegmental area (VTA) specifically express $\alpha 1$ -containing GABA_A receptors, while dopaminergic neurons transmitting to nucleus accumbens from VTA express $\alpha 3$ -containing GABA_A receptors (Fig. 6). Potentiation of $\alpha 1$ -containing GABA_A receptors on GABAergic neurons by benzodiazepines inhibits the neuron and decreases the release of GABA at dopaminergic neuron synapses. This inhibition leads to disinhibition of dopaminergic neurons expressing $\alpha 3$ -containing GABA_A receptors, resulting in more release of dopamine at nucleus accumbens. Theoretically, the effects of benzodiazepines at these two sites would effectively cancel each other out, and thus have nominal effects on dopamine levels. However, the actions of benzodiazepines are more predominant on $\alpha 1$ -containing receptors (Heikkinen et al., 2009), thus dopamine levels are increased. A better understanding of the carisoprodol's interaction at these key GABA_AR isoforms would be very significant in understanding its addictive effects in addition to its therapeutic actions.

Figure I-6. Involvement of GABA_A receptors in drug addiction pathways. **A**, Dopaminergic and GABAergic pathways of mesolimbic system. GABAergic neurons of ventral tegmental area (VTA) specifically express $\alpha 1$ containing GABA_A receptors whereas dopaminergic neurons express $\alpha 3$ containing GABA_A receptors. Dopaminergic neurons transmits to nucleus accumbens and release dopamine. All addictive drugs increase the dopamine levels in nucleus accumbens via mesolimbic pathways. **B**, Benzodiazepines (BZP) mechanism of addiction and likely path followed by carisoprodol (CSP). Potentiation of $\alpha 1$ expressing GABA_A receptors on GABAergic neurons by benzodiazepines or carisoprodol (likely) inhibit the postsynaptic potential and decrease the release of GABA at dopaminergic neuron synapses. Decreased GABA levels at the synapses leads to disinhibition of dopaminergic neurons and increase the dopamine release at nucleus accumbens. Technically BZP (or CSP) should cancel its effects via potentiation of $\alpha 3$ containing GABA_A receptors on dopaminergic neurons but BZP's action is predominant on $\alpha 1$ containing GABA_A receptors.

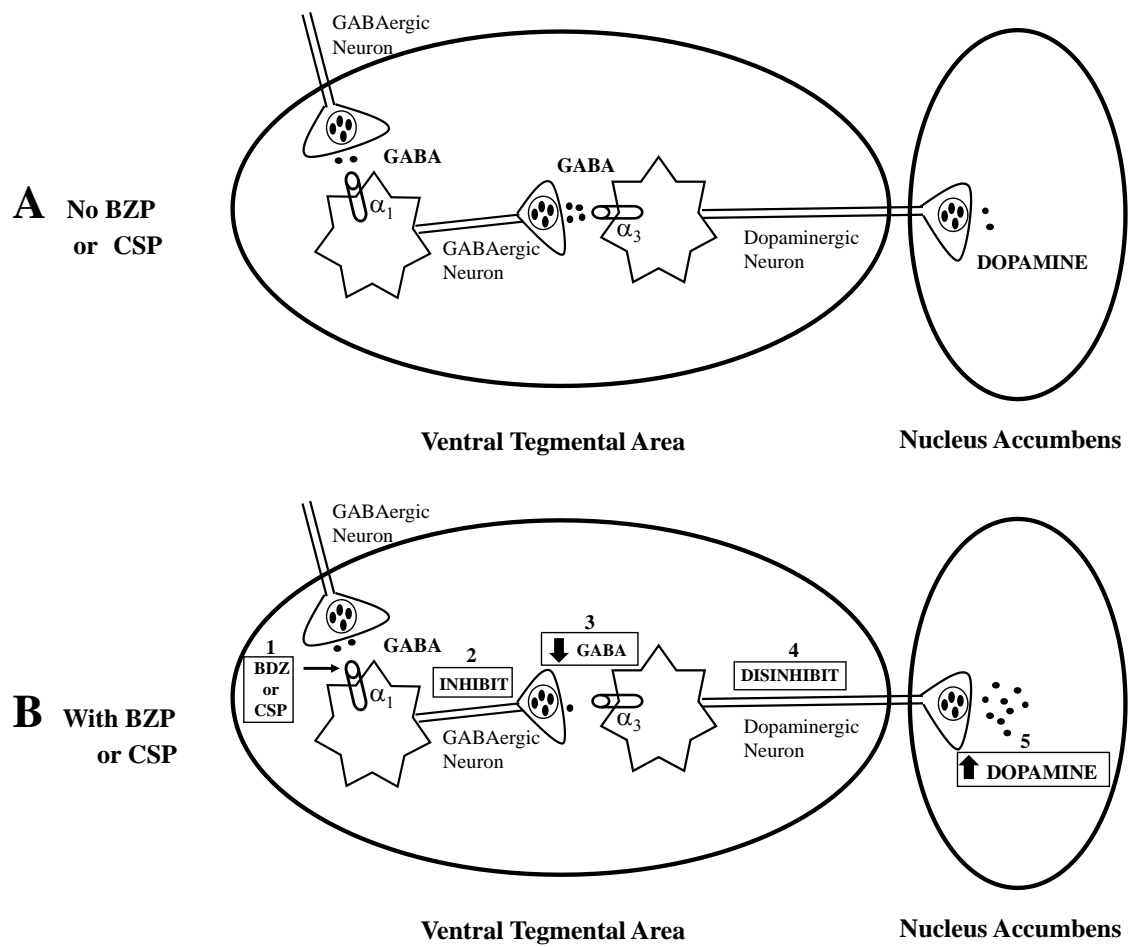


Figure I-6

Objective of the dissertation

In light of the fact that GABA_A receptors subunit isoforms influence physiologic effects like anxiety, sedation, addiction, muscle relaxation and drug tolerance and carisoprodol's therapeutic effects, we **hypothesize that carisoprodol modulates GABA_ARs in a subunit-dependent manner, possibly through a novel site of action, with different amino acid domains contributing to direct gating and allosteric modulation actions of carisoprodol.** Investigation of GABA_AR subunit-dependent effects of carisoprodol and GABA_AR subunit amino acid domains that confer carisoprodol sensitivity would provide critical information for carisoprodol's molecular mechanism of action, that would help in understating the mechanism underlying its abuse potential and muscle relaxing properties and thus, in the treatment of tolerance and withdrawal symptoms of carisoprodol. If carisoprodol is highly efficacious on $\alpha 1$ subunit containing GABA_A receptors, it may be the underlying mechanism for its ability in enhancing the sedative effects of CNS depressants and its high potential for abuse. Moreover, the general anesthetics propofol and etomidate also allosterically modulate and directly gate GABA_ARs through a single site of action whereas distinct GABA_AR sites confer these properties to neurosteroids. A critical question is whether the allosteric and direct gating effects of carisoprodol are mediated via a single or two sites. The aim of the dissertation was divided in two; **1) to identify subunit-dependent influence on direct gating and allosteric enhancement effects of carisoprodol on GABA_ARs and 2) To identify the GABA_ARs subunit domains that confer carisoprodol sensitivity.** To study the subunit-dependent effect of carisoprodol, we transfected HEK 293 cells with desired

receptor configuration and tested for direct activation and allosteric modulation by carisoprodol and to gain insight into possible critical amino acid domains in GABA_AR subunits, we generated site directed mutants of $\alpha 1$ and $\alpha 3$ subunits and tested carisoprodol sensitivity for direct and allosteric effects.

The quest to develop GABAergic ligands with better delineation of therapeutic and adverse actions has begun to show considerable promise in recent years, and a number of agents with unique subunit-selective profiles are in development for the treatment of anxiety, sleep disorder, down syndrome, autism, schizophrenia and epilepsy (Dawson et al., 2005; Rudolph and Mohler, 2014). With regard to muscle relaxants, a drug selective primarily for $\alpha 2$ -expressing GABA_A receptors would likely have less abuse potential and fewer sedative effects (Rudolph and Knoflach, 2011). Considering the fact that low back pain has been one of the top five reasons for physician visits in the United States for decades (Witenko et al., 2014), the need to develop a safe and efficacious agent for this substantial medical need is clear. The overall aim of the study is to gain insight into molecular action of carisoprodol on GABA_ARs that would help us in understanding the mechanism underlie carisoprodol's abuse, muscle relaxant properties and in development of a new drug with better muscle relaxing properties and reduced abuse potential.

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CHAPTER II

ASSESSMENT OF SUBUNIT-DEPENDENT DIRECT GATING AND ALLOSTERIC MODULATORY EFFECTS OF CARISOPRODOL AT GABA_A RECEPTORS

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ABSTRACT

Carisoprodol is a widely prescribed muscle relaxant, abuse of which has grown considerably in recent years. It directly activates and allosterically modulates $\alpha 1\beta 2\gamma 2$ GABA_ARs, although the site(s) of action are unknown. To gain insight into the actions of carisoprodol, subunit-dependent effects of this drug were assessed. Whole-cell patch clamp recordings were obtained from HEK293 cells expressing $\alpha 1\beta 2$, $\alpha 1\beta 3$ or $\alpha x\beta z\gamma 2$ (where $x = 1-6$ and $z = 1-3$) GABA_ARs, and in receptors incorporating the δ subunit (modeling extrasynaptic receptors). The ability to directly gate and allosterically potentiate GABA-gated currents was observed for all configurations. Presence or absence of the $\gamma 2$ subunit did not affect the ability of carisoprodol to directly gate or allosterically modulate the receptor. Presence of the $\beta 1$ subunit conferred highest efficacy for direct activation relative to maximum GABA currents, while presence of the $\beta 2$ subunit conferred highest efficacy for allosteric modulation of the GABA response. With regard to α subunits, carisoprodol was most efficacious at enhancing the actions of GABA in receptors incorporating the $\alpha 1$ subunit. The ability to directly gate the receptor was generally comparable regardless of the α subunit isoform, although receptors incorporating the $\alpha 3$ subunit showed significantly reduced direct gating efficacy and affinity. In extrasynaptic ($\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$) receptors, carisoprodol had greater efficacy than GABA as a direct gating agonist. In addition, carisoprodol allosterically potentiated both EC₂₀ and saturating GABA concentrations in these receptors. In assessing voltage-dependence, we found direct gating

and inhibitory effects were insensitive to membrane voltage, whereas allosteric modulatory effects were affected by membrane voltage. Our findings demonstrate direct and allosteric effects of carisoprodol at synaptic and extrasynaptic GABA_ARs and that subunit isoform influences these effects.

INTRODUCTION

γ -Aminobutyric acid type A receptors (GABA_ARs) are ion channel-coupled, multi-subunit proteins that serve as the primary mediators of inhibitory neurotransmission in the adult central nervous system (CNS). Functional receptors are composed of individual subunits arranged in a pentameric manner. In mammals, the various subunits and their isoforms have been divided into the following classes: α (1-6), β (1-3), γ (1-3), ρ , δ , ϵ , π , and θ (Huang et al., 2006). Subunit architecture is highly conserved among GABA_ARs with each subunit composed of an extracellular amino-terminal, four transmembrane (TM) domains, a large intracellular loop, and an extracellular carboxyl-terminal. Subunit composition determines channel conductance, kinetics, and gating properties of the receptor (Olsen and Sieghart, 2008). Synaptic GABA_ARs are responsible for phasic changes in GABA-mediated post-synaptic inhibition. Extrasynaptic receptors, which typically incorporate a δ subunit (Mortensen et al., 2010), exert a tonic inhibitory influence on neuronal membrane potential. Given their vital role in inhibitory signaling in the CNS, GABA_ARs are the targets of several clinically relevant compounds. These compounds include benzodiazepines, barbiturates, general and inhalational anesthetics, and certain centrally-acting muscle relaxants.

Carisoprodol is a centrally-acting muscle relaxant indicated in the alleviation of acute musculoskeletal conditions (Toth and Urtis, 2004). With a single dose of 350 mg, effects of carisoprodol begin within 30 minutes of administration and plasma concentrations reach 4-7 $\mu\text{g/mL}$ in 2 to 4 hours (Littrell et al., 1993). The dangers associated with carisoprodol

abuse, such as psychomotor impairment and severe withdrawal that may lead to seizures and death, are well-documented (Adams et al., 1975; Elder, 1991; Littrell et al., 1993; Reeves and Parker, 2003; Rust et al., 1993; Zacny et al., 2011, 2012). Precipitated withdrawal studies in mice have demonstrated tolerance to carisoprodol develops in as few as four doses (Gatch et al., 2012). In recent years, the ready availability of carisoprodol via internet pharmacies has led to increased recreational use of carisoprodol. Because of the increasing misuse of carisoprodol and associated adverse effects, the United States Drug Enforcement Administration placed carisoprodol into Schedule IV of the Controlled Substances Act in January 2012.

The illicit effects of carisoprodol are generally attributed to the actions of its primary metabolite, meprobamate—a federally controlled substance with barbiturate-like activity at GABA_ARs (Rho et al., 1997). While conversion to meprobamate likely contributes to the therapeutic and illicit effects of carisoprodol, the pharmacological and physiological profiles of carisoprodol are not entirely consistent with that of its metabolite, supporting the possibility that carisoprodol may have effects independent of meprobamate.

The full spectrum of potential targets of carisoprodol has not been established. Adverse effects may be associated with serotonergic-like effects (Bramness et al., 2004). In animal studies, the NMDA receptor antagonist dizocilpine partially substitutes for the discriminative stimulus effects of carisoprodol (M. Gatch, personal communication). Considerable evidence implicates the GABA_AR in its effects. As noted, the metabolite of carisoprodol has barbiturate-like effects at GABA_ARs (Rho et al., 1997). We have

demonstrated carisoprodol itself allosterically modulates and directly activates human $\alpha 1\beta 2\gamma 2$ GABA_ARs, and its actions are not mediated via reported sites of action for benzodiazepines or barbiturates (Gonzalez et al., 2009b). Although receptors of $\alpha 1\beta 2\gamma 2$ subunit composition are the prevalent configuration in the brain, a vast array of GABA_AR configurations have been shown to exist throughout the CNS, with each configuration contributing to specific physiological and pharmacological responses (Olsen and Sieghart, 2008). For example, benzodiazepines mediate sedative and anticonvulsant effects through $\alpha 1$ -containing GABA_ARs, anxiolytic effects primarily through $\alpha 2$ -expressing receptors, and myorelaxant effects via receptors expressing $\alpha 3$ and $\alpha 5$ subunits (Tan et al, 2010). It has also been demonstrated that abuse and dependence potential of benzodiazepines and barbiturates are related to their subunit-selective interactions with GABA_ARs (Ator, 2005; Ito et al., 1996; Licata and Rowlett, 2008; Wafford, 2005). Because dependence and withdrawal symptoms are associated with carisoprodol use, a better understanding of its molecular mechanism is needed. Thus, we assessed direct and allosteric actions of carisoprodol in varying configurations of synaptic and extrasynaptic GABA_A receptors. Given the clinical and adverse effects associated with carisoprodol, we hypothesized it would interact with a number of GABA_ARs in addition to $\alpha 1\beta 2\gamma 2$, likely those expressing $\alpha 2$ and/or $\alpha 3$ and/or $\alpha 5$ subunits. Our results indicate that the actions of carisoprodol are influenced by subunit isoforms and that direct and allosteric effects are likely mediated via distinct domains.

MATERIALS AND METHODS

Cell Culture and Transfection

Both stably- and transiently-expressing GABA_A receptors HEK293 cells were used in the present study. Human embryonic kidney 293 (HEK293) cells were transfected with human GABA_A α 1- α 6; human β 1-2; and human γ 2s (short isoform) cDNA in a 1:1:5 ratio using *TransIT*[®]-293 (Mirus Bio, Madison, WI) and used for recording 24-48 h later. The γ 2s subunit will be referred to as γ 2 from this point forward. Human GABA_A α 1 subunit cDNA was generously provided by Neil Harrison (Columbia University Medical Center, New York). In resequencing the γ 2 subunit that was used in many of these studies, we detected a mutation (D to N at position 115). We mutated this residue to the native form (QuikChange[®] Site-Directed Mutagenesis Kit, Agilent Technologies, Santa Clara, CA), confirmed the mutation via sequencing, and conducted a number of studies to assess possible impact on carisoprodol activity. We observed no effect and thus data with that mutation are included. HEK293 stably expressing human α 1 β 2 γ 2 or α 2 β 2 γ 2 GABA_ARs were also used. A complete description of the preparation and maintenance of these stable cell lines has been published previously (Hawkinson et al., 1996). For studies assessing carisoprodol effects in a model of extrasynaptic receptors (rat α 1 β 3 δ and α 4 β 3 δ subunits) a transfection ratio of 2:1:0.25 for α : β : δ plasmids was used, as this transfection ratio reliably results in receptors expressing the native 2 α :2 β :1 δ stoichiometry (Wagoner and Czajkowski, 2010). The rat GABA_A α 4 subunit cDNA was purchased from Genescript (Piscataway, New Jersey). As rat extrasynaptic GABA_ARs have been shown to have

similar physiology and pharmacology to human extrasynaptic GABA_ARs (Adkins et al., 2001; Feng et al., 2004; Feng and Macdonald, 2010; Mortensen et al., 2010), use in these studies is appropriate. For all studies, cells were plated on glass coverslips coated with poly-L-lysine in 35-mm culture dishes. Cells were incubated and maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂.

Electrophysiology

Whole-cell patch clamp electrophysiology was used to assess GABA- or carisoprodol-activated Cl⁻ currents. Except for studies examining voltage-dependence (below), electrophysiology experiments were conducted at room temperature (22-25°C) with the membrane potential clamped at -60 mV. Patch pipettes of borosilicate glass (1B150F; World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC; Sutter Instrument Company, Novato, CA) to a tip resistance of 4–6MΩ. Patch pipettes were filled with a solution consisting of 140 mM CsCl, 10 mM EGTA-Na⁺, 10 mM HEPES-Na⁺, and 4 mM Mg²⁺-ATP, pH 7.2. Coverslips containing cultured cells were placed in the recording chamber on the stage of an inverted light microscope and superfused continuously with an external solution consisting of 125 mM NaCl, 20 mM HEPES, 3 mM CaCl₂, 5.5 mM KCl, 0.8 mM MgCl₂, and 10 mM glucose, pH 7.3. Agonist-induced Cl⁻ currents were obtained with an Axopatch 200B amplifier with a rate of 50 samples per second (Molecular Devices, Sunnyvale, CA) equipped with a CV-203BU headstage. Currents were low-pass filtered at 5 kHz, monitored simultaneously on an

oscilloscope and a chart recorder (Gould TA240; Gould Instrument Systems Inc., Cleveland, OH), and stored on a computer using an on-line data acquisition system (pCLAMP 6.0; Axon Instruments) for subsequent off-line analysis

Chemicals and solutions

Carisoprodol, pentobarbital, THIP (4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol), salts and buffers were purchased from Sigma Aldrich (St. Louis, MO). GABA was obtained from Acros Organics (New Jersey, US). GABA stock solution (500 mM) was prepared in de-ionized water. A stock solution (1 or 3M) of carisoprodol and THIP (1M) was made in DMSO. Final concentrations of DMSO in working solutions were 0.3% or less, a concentration that does not affect GABA-gated current. Diazepam and furosemide were utilized from a stock solution prepared in water. All stock solutions were stored at -20°C. On experimental days, drug-containing solutions were prepared from stock by serial dilution into external solution.

Experimental protocol

GABA (with or without carisoprodol) or carisoprodol were applied to each cell by gravity flow using a Y-shaped tube positioned adjacent to the cell. The modulatory effects of carisoprodol on GABA-gated currents were assessed using an EC₂₀ gating concentration of GABA as the control. This gating concentration was selected to ensure there was a sufficient range to observe the full allosteric potential of carisoprodol. To ensure the gating

concentration was approximately an EC₂₀, control responses were compared to the maximal GABA-gated current for each individual cell. Carisoprodol was tested only if the gating concentration was within the EC₁₅₋₂₅ range. Control responses were established by observing two consecutive agonist-activated currents that varied in amplitude by no more than $\pm 10\%$. In our analyses of the modulatory effects of carisoprodol, peak current amplitude was defined as the maximum current elicited by carisoprodol. In studies investigating direct gating effects, carisoprodol-gated currents were normalized to maximum GABA-mediated current. In recordings where an inhibitory component was observed (high CSP concentrations), a rebound current was sometimes followed the inhibitory phase. The channel state(s) from which these openings occur may vary (Feng et al., 2004; Williams et al., 1997; Wooltorton et al., 1997), and thus peak current amplitude was taken during the active drug application phase. For $\alpha\beta\gamma$ configurations, incorporation of $\gamma 2$ subunit was confirmed in $\alpha 1$ -, $\alpha 2$ -, $\alpha 3$ - and $\alpha 5\beta 2\gamma 2$ GABA_ARs by demonstration of sensitivity of GABA (EC₅₀)-gated currents to allosteric potentiation by diazepam (1 μ M). Antagonism by furosemide (100 μ M) was used to confirm expression of $\alpha 4\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ GABA_ARs. Presence of the δ subunit in $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ receptors was confirmed by loss of inhibition by 1 μ M Zn²⁺ on GABA-gated current. After establishing the control response, effects of the test drugs were determined.

To study the voltage-dependence effect of carisoprodol in GABA_A receptors, $\alpha 1\beta 2\gamma 2$ transfected HEK293 cells were clamped at -60 mV and +60 mV and studied for carisoprodol direct gating, allosteric modulatory and blocking effects as described above.

Data Analysis

To ensure equipotent concentrations were used for gating, GABA concentration-response data were collected for all synaptic and extrasynaptic GABA_ARs tested, (Table 1 and 4). From these data, EC₂₀ and saturating GABA concentrations were calculated for each configuration and used in subsequent investigations of the allosteric and direct effects of carisoprodol, respectively.

Concentration-response profiles for the positive modulatory actions of carisoprodol were generated (Origin; OriginLab Corp., Northampton, MA) using the equation $I/I_{\max} = [\text{carisoprodol}]^n / ([\text{carisoprodol}]^n + \text{EC}_{50}^n)$, where I is the normalized current amplitude at a given concentration of carisoprodol, I_{\max} is the maximum current induced by carisoprodol, EC₅₀ is the half-maximal effective concentration of carisoprodol, and n is the Hill coefficient. For concentration-response curves illustrating allosteric actions, a correction was applied to subtract direct gating effects. In some cases, the blocking actions of carisoprodol became notable at high concentrations; in these instances, curves were fitted to the data point corresponding to peak effect, and the curve was extrapolated.

All data are presented as mean values \pm S.E.M. Statistical significance ($p < 0.05$) between control and test conditions was determined using Student's t -test (paired or unpaired) and one-way analysis of variance. Tukey-Kramer *post hoc* test for multiple comparisons was performed as needed.

RESULTS

Influence of α subunit isoform of carisoprodol-mediated activity of the GABA_A receptor

α subunit influences on direct gating by carisoprodol. Table 1 provides EC₅₀ values for the synaptic GABA_A receptors studied in the present investigation. Both allosteric (assessed using an EC₂₀ GABA gating concentration) and direct effects of carisoprodol (1 μ M – 10 mM) were assessed in α x β 2 γ 2 GABA_A receptors, where x = α subunit 1-6. Carisoprodol directly gated each of the configurations tested, evoking inward currents in the absence of GABA (Fig. 1A and 1B). Maximal carisoprodol-gated currents were of similar magnitude regardless of α subunit, with efficacies ranging from 13-43% of maximal GABA-gated current (Fig. 1B, Table 2). However, carisoprodol direct gating potency and efficacy was significantly less at α 3-containing receptors compared to other α isoform receptors (Table 2). As originally reported for α 1-containing receptors (Gonzalez et al., 2009b), attenuation of current amplitude at high concentrations, followed by rebound current, was consistently observed regardless of the α subunit.

α subunit influences on allosteric modulation by carisoprodol of GABA-gated current. We also assessed the ability of carisoprodol to allosterically modulate the response to GABA when α subunits were varied. Carisoprodol positively modulated the effects of an EC₂₀ concentration of GABA in all configurations tested (Fig. 2A and 2B). Carisoprodol potency was not significantly influenced by the α subunit isoform (Table 3) whereas the efficacy of carisoprodol modulation was influenced by the α subunit, being

greatest in $\alpha 1$ -containing receptors (Fig. 2B and Table 3). As observed when evaluating direct gating effects of carisoprodol, allosteric enhancement began to diminish with higher concentrations of carisoprodol, and a notable rebound current was observed. Pharmacologically, the presence of carisoprodol increased the apparent affinity of GABA, resulting in a significant leftward shift in the GABA concentration-response profile without increasing maximal GABA current amplitude (Fig. 2E).

Influence of β subunit isoform of carisoprodol-mediated activity of the GABA_A receptor

β subunit influences on direct gating by carisoprodol. As we reported previously (Gonzalez et al., 2009a), application of carisoprodol to $\alpha 1\beta 2\gamma 2$ receptors elicited inward currents in the absence of GABA, with an efficacy approximately 43% of that observed in response to a saturating concentration of GABA (Fig. 1A). The ability to directly gate the receptor was also observed in $\alpha 1\beta 1\gamma 2$ GABA_ARs. Efficacy in $\beta 1$ -containing receptors was significantly greater than that observed in $\beta 2$ -containing receptors (Fig. 1C and Table 2) and approximated 70% of the magnitude of maximal GABA-gated current in $\alpha 1\beta 1\gamma 2$ GABA_ARs. Potency of carisoprodol's direct gating effect was similar in $\beta 1$ - and $\beta 2$ -expressing receptors (Table 2).

β subunit influences on allosteric modulation by carisoprodol of GABA-gated current. Carisoprodol modulated the GABA-gated currents of $\alpha 1\beta 1\gamma 2$ GABA_ARs in a manner previously described for $\alpha 1\beta 2\gamma 2$ GABA_ARs—potentiation in a concentration-

dependent manner accompanied by inhibition and rebound currents at millimolar concentrations (Fig. 2A). The β subunit isoform affected both potency ($33.1 \pm 4 \mu\text{M}$ and $88.2 \pm 20 \mu\text{M}$, in $\beta 1$ - and $\beta 2$ -containing receptors, respectively) and efficacy (maximum efficacy was $225 \pm 14.6\%$ of control in $\beta 1$ -containing receptors, compared to $474.7 \pm 53.5\%$ in $\beta 2$ -containing receptors) of carisoprodol-mediated potentiation of GABA-gated currents (Fig. 2C and Table 3). This is in contrast to the pattern observed for the direct gating effects of carisoprodol, in which carisoprodol was more efficacious at receptors containing the $\beta 1$ isoform.

Direct gating and allosteric modulatory effects of carisoprodol do not require the γ subunit

In assessing the influence of the γ subunit on actions of carisoprodol, we found its efficacy as a direct agonist was unchanged with the presence of the γ subunit (Fig. 1A and 1D, Table 2). Peak current amplitude of carisoprodol-evoked currents was $45.9 \pm 3\%$ of the maximum GABA-gated current for $\alpha 1\beta 2$ receptors, whereas it was $43.6 \pm 4.3\%$ for $\alpha 1\beta 2\gamma 2$ receptors. Consistent with this, we observed the actions of carisoprodol were comparable regardless of whether the $\gamma 2\text{short}$ or $\gamma 2\text{long}$ isoform was expressed (data not shown).

We also assessed the influence of the γ subunit on allosteric actions of carisoprodol. Micromolar concentrations of carisoprodol potentiated the GABA-gated currents of $\alpha 1\beta 2$

GABA_ARs in a concentration-dependent manner (Fig. 2A and 2D, Table 2). The patterns of potentiation and inhibition by carisoprodol at $\alpha 1\beta 2$ GABA_ARs were similar to those observed at $\alpha 1\beta 2\gamma 2$ GABA_ARs, shown here (Fig. 1A) and previously using a stable $\alpha 1\beta 2\gamma 2$ cell line (Gonzalez et al., 2009b). The estimated EC₅₀ for carisoprodol at $\alpha 1\beta 2$ GABA_ARs was $87.4 \pm 16.4 \mu\text{M}$ compared to $88.2 \pm 20 \mu\text{M}$ for receptors containing the $\gamma 2$ subunit, with direct gating by carisoprodol likely contributing to maximal current amplitude elicited by higher concentrations of the drug. Maximum potentiation of control currents occurred with 1 mM carisoprodol for each configuration (Fig. 1D), with efficacies of $346.7 \pm 67.6\%$ and $474.7 \pm 53.5\%$ for $\alpha 1\beta 2$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs, respectively. Thus, the γ subunit did not significantly influence the potency or efficacy of carisoprodol as an allosteric modulator (Table 2). At millimolar concentrations, rebound currents were observed upon termination of drug application, and co-application of 3 mM carisoprodol elicited an inhibitory effect on GABA-gated currents during drug application. Together with the direct gating studies, these findings demonstrate the γ subunit is not essential for carisoprodol-mediated regulation of GABA_AR function.

Effects of carisoprodol in “extrasynaptic” receptors

δ subunit influences on direct gating by carisoprodol. To assess carisoprodol mediated-activity in a model of extrasynaptic GABA_A receptors, we studied its effects in HEK293 expressing rat $\alpha 1$ or $\alpha 4$, $\beta 3$ and δ GABA_A receptor subunits. Carisoprodol was more efficacious than GABA in directly gating $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ extrasynaptic receptors,

with the latter receptor having the highest sensitivity to CSP (Fig. 3, Table 4). We also compared the efficacy of carisoprodol to that of THIP, which has high efficacy at extrasynaptic GABA_A receptors (Belelli et al., 2009; Brickley and Mody, 2012). As reported by others, we observed that THIP was a super agonist, with THIP-gated currents being 135.6 ± 7.2 and $153.4 \pm 10.7\%$ of the amplitude of currents obtained with saturating GABA in $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ receptors, respectively. Carisoprodol was thus roughly 90% as efficacious as THIP in directly gating $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ receptors (Fig. 3). Direct gating efficacy by carisoprodol in rat $\alpha 1\beta 2$, $\alpha 1\beta 3$, and $\alpha 1\beta 3\gamma 2$ configurations was similar, indicating neither the $\beta 2/3$ isoforms nor the γ subunit significantly affects direct activation by carisoprodol. Effects in the rat $\alpha 1\beta 2$, $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ receptors were also generally comparable to what we observed across a range of human GABA_A receptors (see Fig. 1). Thus, species differences do not appear to be a significant influence with regard to direct gating effects of carisoprodol, as has been observed for other GABAergic ligands such as pentobarbital, neurosteroids, THIP and general anesthetics (Adkins et al., 2001; Feng et al., 2004; Feng and Macdonald, 2010; Mortensen et al., 2010).

δ subunit influences on allosteric modulation by carisoprodol of GABA-gated current. We also determined the ability of carisoprodol to allosterically modulate GABA-gated current in extrasynaptic receptors. In contrast to what was observed with direct gating, the δ subunit did not have a significant effect on allosteric modulatory effects of carisoprodol (Fig. 4). These results also confirm carisoprodol is most efficacious on receptors expressing $\beta 2$ compared to $\beta 1$ or $\beta 3$ subunits for its allosteric modulatory effects

(Table 4). Interestingly, like pentobarbital, neurosteroids, and general anesthetics (Feng et al., 2004; Meera et al., 2009; Wohlfarth et al., 2002), carisoprodol potentiated maximal GABA currents of $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ GABA_A receptors, but not of $\alpha 1\beta 3$ or $\alpha 1\beta 3\gamma 2$ receptors (Fig. 5). These results demonstrate carisoprodol has distinct gating properties on extrasynaptic GABA_A receptors compared to synaptic receptors.

Effect of membrane voltage on direct gating and allosteric modulatory effects of carisoprodol

As voltage sensitivity may sometimes provide insight into possible site of action of a ligand, we determined whether direct gating, allosteric modulatory and/or blocking actions of carisoprodol are voltage-dependent, by assessing these actions in $\alpha 1\beta 2\gamma 2$ GABA_A receptors with the membrane voltage clamped at -60 mV and +60 mV. GABA-gated and CSP-gated current at 0 mV was essentially undetectable, consistent with the theoretical reversal potential of 0.4 mV, as predicted from the Nernst equation. As reported by others (O'Toole and Jenkins, 2012), we observed outward rectification in response to lower concentrations of GABA (current amplitude at +60 mV was 110% of that recorded at -60 mV), whereas no significant rectification was observed at saturating GABA. To assess voltage-dependence of the direct gating effects of CSP, we chose a concentration of 1 mM, which elicits a robust current, but nominal apparent inhibition. As shown in Figure 6 (A, B), the direct gating effect of CSP was not impacted by membrane voltage. Interestingly, allosteric potentiation of GABA currents by carisoprodol was affected by cell

membrane polarity; in these studies, 100 μ M CSP enhanced GABA current to $285.3 \pm 17.7\%$ of control at -60 mV, whereas the extend of potentiation was $204.8 \pm 5.3\%$ at +60 mV (Fig. 6C, 6D). To assess voltage-dependence of blocking by carisoprodol, we compared the decrease in current amplitude in response to 5 mM CSP, compared to 3 mM CSP, at both -60 and +60 mV. The magnitude of current attenuation with 5 mM CSP was similar in both cases (Fig. 6E, 6F).

DISCUSSION

Despite the clinical utility of carisoprodol in treatment of low back pain, its use is complicated by its abuse potential. Carisoprodol abuse, tolerance, and withdrawal are well-documented in the literature (Adams et al., 1975; Elder, 1991; Reeves and Parker, 2003; Rust et al., 1993). This abuse has become particularly apparent in recent years, due in part to ready access of carisoprodol via internet pharmacies. The abuse liability of carisoprodol has often been attributed to meprobamate—the primary metabolite of carisoprodol and a controlled substance at the federal level. Our previous research has demonstrated that carisoprodol itself, independent of its conversion to meprobamate, acts at GABA_ARs in a manner described for drugs of abuse that act via the GABAergic system (Gonzalez et al., 2009b). Whereas meprobamate has been federally scheduled for many years, such was not the case for carisoprodol. Effective January 2012, following extensive review of scientific research and data on abuse, law enforcement encounters, and other information, the United States Drug Enforcement Agency placed carisoprodol into Schedule IV of the Controlled Substances Act (Federal Register, vol 76, no 238, pp. 77330-77360, Dec 12, 2011).

The abuse and dependence potential of many GABAergic drugs are related to their subunit-selective interactions with GABA_ARs (Ator, 2005; Ator et al., 2010; Ito et al., 1996; Licata and Rowlett, 2008; Wafford, 2005). Thus, we assessed whether the actions of carisoprodol are subunit-dependent, potentially underlying its physiological effects and abuse liability. The role of the γ subunit has been established for several modulators of GABA_AR function. The most prominent example is the benzodiazepine class of drugs,

which require the presence of a γ subunit to potentiate GABA-gated currents (Pritchett et al., 1989). The γ subunit does not play an essential role in mediating the actions of carisoprodol at GABA_ARs because the modulatory and agonistic effects of the drug were not significantly different between $\alpha 1\beta 2$ and $\alpha 1\beta 2\gamma 2$ configurations. These findings support our earlier assertion that carisoprodol does not act at the benzodiazepine site of the receptor (Gonzalez et al., 2009a) and led us to pursue the α and β subunits as critical mediators of the actions of carisoprodol.

In the current study, the influence of β subunit isoforms was examined via comparison of effects elicited from $\beta 1$ -, $\beta 2$ - and $\beta 3$ -containing receptors. Whereas the actions of GABAergic compounds such as etomidate, loreclezole, and furosemide are dependent upon incorporation of $\beta 2$ or $\beta 3$ subunits (Korpi et al., 2002; Korpi et al., 1995; Olsen, 2014; Stewart et al., 2013), carisoprodol-mediated effects were observed at $\alpha 1\beta 1\gamma 2$ GABA_ARs, suggesting critical domains for carisoprodol activity are located within regions conserved between the $\beta 1$ and $\beta 2$ isoforms. For allosteric modulation, carisoprodol was significantly more efficacious at $\beta 2$ -containing receptors. Expression of the $\beta 2$ isoform in the brain is extensive (Sigel and Steinmann, 2012), suggesting the majority of receptor configurations are susceptible to modulation by carisoprodol. In contrast, direct activation by carisoprodol was significantly greater at $\beta 1$ -containing receptors. This disparity suggests allosteric modulation and direct gating by carisoprodol may be mediated by distinct sites of the β subunit.

There has been considerable progress in recent years in understanding the involvement of specific GABA_A receptor α subunits in both desired and adverse pharmacologic actions (for review, see (Rudolph and Mohler, 2006). α 1-expressing GABA_A receptors are critical for the sedative effects of benzodiazepines (McKernan et al., 2000) whereas α 2- and/or α 3-expressing receptors underlie their anxiolytic actions (Atack et al., 2005; Dias et al., 2005). In addition, the efficacy of benzodiazepines at α 1-containing GABA_ARs may predict their abuse potential (Ator, 2005; Licata and Rowlett, 2008). Inhibitory GABAergic interneurons in the ventral tegmental area (VTA) specifically express α 1-containing GABA_ARs (Tan et al., 2010). Potentiation of α 1-containing GABA_ARs on these interneurons causes disinhibition of VTA dopaminergic neurons, resulting in increased dopamine release in the nucleus accumbens. Addictive drugs increase dopamine levels in the mesolimbic system (Luscher and Ungless, 2006). As physical dependence is more likely to develop with drugs that interact with a broader collection of GABA_AR subtypes (Licata and Rowlett, 2008), the high efficacy of carisoprodol for both direct and allosteric effects on α 1-containing receptors, coupled with its ability to interact with GABA_AR subtypes, is consistent with its high abuse potential (Rudolph and Knoflach, 2011).

The therapeutic goal of carisoprodol use is alleviation of low back pain, which results from centrally-mediated muscle relaxation. Others have demonstrated muscle relaxation is associated preferentially with α 2- and α 3-expressing receptors (Crestani et al., 2001; Griebel et al., 2003; Licata et al., 2005; Licata et al., 2009). Although

carisoprodol did not selectively interact with these subunits, it had notable allosteric modulatory effects on both $\alpha 2$ - and $\alpha 3$ -expressing receptors and almost similar potency for direct activation for $\alpha 2$ -containing receptors as compared to most abundant configuration of human CNS, $\alpha 1$ -containing receptors (Olsen and Sieghart, 2008). Taken together, the pharmacological profile of carisoprodol is consistent with its clinical effects and its potential for abuse. A molecule that selectively interacts with $\alpha 2/\alpha 3$ GABA_A receptor subunits would seem promising as a muscle relaxant with reduced abuse potential and sedative effects (Rudolph and Knoflach, 2011).

Unlike benzodiazepines, carisoprodol also has the ability to directly gate the GABA_AR. Misuse of agents that directly gate the receptor is more likely to result in death than those that only allosterically enhance receptor activity, and overdose of carisoprodol is responsible for a substantial number of both unintentional and intentional deaths (Fass, 2010). We observed that carisoprodol elicited significant and concentration-dependent direct gating effects at the GABA_AR. According to case reports, blood or plasma concentrations of carisoprodol as low as 140 μ M have proven to be fatal (McIntyre et al., 2012; Robertson and Marinetti, 2003). Interestingly, this concentration correlates well to the onset of direct activation for the majority of receptor subunit complexes tested in the present study.

These studies are the first to explore subunit-dependent actions of carisoprodol, and thus little is known about possible domains that are key for its actions. One fundamental question is whether the site for allosteric and direct gating effects is the same. The general

anesthetics propofol and etomidate also both allosterically enhance and directly gate the receptor, and evidence suggests a single site of action confers both properties (Forman, 2011; Ruesch et al., 2012). In contrast, the ability of neurosteroids to directly gate and allosterically modulate the receptor is mediated via distinct sites (Hosie et al., 2006). Our data are most consistent with the contention that the two actions of carisoprodol are mediated via distinct sites. First, we observed greatest direct gating efficacy in $\beta 1$ -expressing receptors, whereas efficacy for allosteric modulation was greatest in $\beta 2$ -expressing receptors. Similarly, we observed decreased potency and efficacy for direct gating capacity in $\alpha 3$ -expressing receptors, whereas allosteric effects of those receptors were similar to other α subunit-expressing receptors.

We also studied the actions of carisoprodol on δ subunit-containing extrasynaptic GABA_A receptors, which exert a tonic inhibitory tone as opposed to the phasic inhibition characteristic of synaptic receptors (Glykys and Mody, 2007; Herd et al., 2009). It should also be noted that $\alpha 5\beta 2\gamma 2$ receptors, in addition to a role at synapses (Serwanski et al., 2006), are present at extrasynaptic sites and have a prominent role in contributing to tonic inhibition (Caraiscos et al., 2004; Glykys and Mody, 2006). The involvement of extrasynaptic GABA_A receptor dysfunction in neurological disorders like depression (Maguire et al., 2005), schizophrenia (Maldonado-Aviles et al., 2009), and some forms of epilepsy (Cope et al., 2009) has made these receptors an emerging clinical target (Brickley and Mody, 2012). As GABA has high affinity but low efficacy at extrasynaptic GABA_A receptors, it is considered a partial agonist at these receptors (Bianchi and Macdonald,

2003). We found that, as reported for other ligands (Bianchi and Macdonald, 2003), carisoprodol has higher efficacy at extrasynaptic $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ receptors than does GABA, and carisoprodol was about 90% as efficacious as the full agonist THIP. The efficacy of general anesthetics and sedative/hypnotics in potentiation of GABA-gated currents at extrasynaptic $\alpha 6\beta 2\delta$ GABA_A receptors depends upon the ambient GABA concentration or GABA occupancy of the receptors (Houston et al., 2012). The general anesthetic propofol and sleep-inducing drug THIP potentiate only when the ambient GABA levels are low and cease to modulate at saturating GABA. On the other hand, the neurosteroid THDOC (3 α ,21-dihydroxy-5 α -pregnan-20-one) has stronger potentiating effects on saturated GABA currents compared to submaximal GABA currents (Houston et al., 2012; Wohlfarth et al., 2002). We found that carisoprodol potentiated at both sub-saturating and saturating GABA concentrations. This is also true for pentobarbital (Feng et al., 2004). The N-terminal domain of the δ subunit has been shown to be involved in pentobarbital potentiation of saturated GABA currents of $\alpha 1\beta 3\delta$ receptors (Feng and Macdonald, 2010). The profile of carisoprodol at both synaptic and extrasynaptic GABA_AR is similar to that of barbiturates, although equivalent domains for action at synaptic receptors are unlikely (Gonzalez et al., 2009b). Whether the N-terminal domains of the δ subunit that influence pentobarbital potentiation of saturated GABA current also influence carisoprodol sensitivity remains to be determined.

At high concentrations, carisoprodol also inhibits GABA_AR function; this is evidenced by decreasing current amplitude and/or rebound currents associated with

termination of drug application. A similar phenomenon also exists for barbiturates and gamma-butyrolactones (Akk et al., 2004; Feng et al., 2004; Rho et al., 1996; Williams et al., 1997; Wooltorton et al., 1997). This rebound current has been explained as reentry into an active state following desensitization (Wooltorton et al., 1997), or as relief from ligand binding to a low affinity inhibitory site, which permits reopening of the channel (Akk et al., 2004; Feng et al., 2004; Rho et al., 1996; Williams et al., 1997). In the case of carisoprodol, rebound from block seems most likely. We have reported in abstract form (Kumar and Dillon, 2014) that in wild type homomeric $\beta 3$ GABA receptors, which are spontaneously open, application of carisoprodol by itself blocks the spontaneously open channels. In homomeric receptors with introduction of a transmembrane domain 2 mutation (mutation of the 6' T to F), blockade of the spontaneously open current is eliminated, and carisoprodol instead activates a significant inward current. Thus rebound currents following high concentrations of carisoprodol appear to be due to CSP dissociation from a low affinity inhibitory site. We did not fully quantitate the inhibitory effect, as it likely play a nominal role in the therapeutic or adverse effects of carisoprodol, and its low affinity combined with drug solubility issues makes accurate quantitation of it difficult.

We also studied whether the actions of carisoprodol on GABA_A receptors display voltage-dependence. Neither direct gating nor blocking effects were affected by transmembrane voltage. However, we found allosteric modulatory effects of carisoprodol were significantly reduced at + 60 mV compared to -60 mV; a similar effect has been reported for the general anesthetics etomidate, propofol and isoflurane, (O'Toole and

Jenkins, 2012). One might conclude the differential voltage-dependence of direct and allosteric effects supports the likelihood of two sites for these effects. Whereas collectively our data do support distinct sites, the differences in voltage-dependence for direct and allosteric effects do not necessarily lead to this conclusion. The reduced effect of carisoprodol at + 60 mV may not relate to voltage per se, but instead is likely related to the enhanced open probability (P_o) of the channel when clamped at positive potentials, as effects of allosteric potentiators are inversely related to P_o (O'Toole and Jenkins, 2012).

With regard to lack of voltage-dependence of the inhibitory effect, one might conclude a site of action in the channel is unlikely. Whereas the presence of voltage-dependence of block is consistent with a site of action in the channel, it is not a requirement for a channel blocking mechanism. Indeed, the prototypical GABA_A blocker, picrotoxin, does not display voltage-dependence (Newland and Cull-Candy, 1992; Yoon et al, 1993), and there is a considerable body of evidence that picrotoxin binds in the ion channel (see Bali and Akabas, 2007, and several references therein). The fact that mutation of the TM2 6' residue does impact carisoprodol-mediated inhibition (Kumar and Dillon, 2014) is consistent with a site of action within the channel, but definitive conclusions regarding the site for inhibitory effects of carisoprodol will require additional study.

The recent scheduling at the federal level of carisoprodol confirms the danger this drug poses when misused and abused. In the current study, we demonstrated carisoprodol preferentially interacts with selective GABA_AR subunits. The pharmacological profile of carisoprodol at GABA_ARs we have identified is consistent with the therapeutic effects of

the drug, and its subunit-selective actions may underlie its potential for abuse. Moreover, the complex interactions of carisoprodol suggest it interacts with multiple sites on the receptor. To our knowledge, no other GABAergic ligand has a subunit-dependent profile equivalent to that of carisoprodol, suggesting this drug may be acting at a novel site.

Table II-1. GABA sensitivity of different GABA_A receptors subunit configurations.
GABA EC₅₀ values and Hill coefficients were calculated from each receptor configuration as mean \pm S.E.M. from n cells.

Receptor Configuration	EC₅₀ (μM)	Hill Coefficient	n
α1β2	14.0 ± 1.01	1.32 ± 0.11	4
α1β2γ2	35.5 ± 0.64	1.32 ± 0.03	6
α2β2γ2	48.4 ± 5.71	1.09 ± 0.12	9
α3β2γ2	34.8 ± 2.09	1.04 ± 0.06	8
α4β2γ2	4.48 ± 0.29	1.36 ± 0.11	6
α5β2γ2	2.50 ± 0.20	1.11 ± 0.09	6
α6β2γ2	0.47 ± 0.06	1.02 ± 0.12	8
α1β1γ2	6.6 ± 1.07	1.17 ± 0.09	6

TABLE II-1.

Table II-2. Comparison of potency and efficacy of carisoprodol as a direct gating agonist at different GABA_A receptor subunit configurations. Carisoprodol EC₅₀ was determined relative to maximum current elicited by carisoprodol. Carisoprodol efficacy is normalized to maximum GABA-gated currents. The potency and efficacy of carisoprodol was significantly less at α 3-containing receptors relative to other α - subunit isoforms of GABA_A receptors. Each data point represents the mean \pm S.E.M from n cells. Significant differences compared to α 1 β 2 γ 2 receptor are denoted by *, p < 0.05; **, p < 0.01.

Receptor Configuration	EC₅₀ (μM)	Efficacy (%)	n
α1β2	679.3 ± 24.3	45.9 ± 3.0	9
α1β2γ2	685.5 ± 30.4	42.6 ± 4.3	31
α2β2γ2	829.4 ± 50.2	30.1 ± 1.4	9
α3β2γ2	1867.6 ± 92.4*	13.4 ± 1.4**	29
α4β2γ2	757.7 ± 61.3	37.3 ± 5.0	8
α5β2γ2	834.7 ± 60.3	37.7 ± 3.5	9
α6β2γ2	294.3 ± 28.1	36.8 ± 4.7	12
α1β1γ2	728.8 ± 58.1	69.7 ± 5.4*	12

TABLE II-2.

Table II-3. Comparison of potency and efficacy of allosteric effects of carisoprodol at different GABA_A receptor subunit configurations. EC₅₀ for allosteric modulation by carisoprodol was calculated in the presence of GABA (EC₂₀). Efficacy was calculated as maximum current elicited by carisoprodol plus GABA (EC₂₀) normalized to GABA (EC₂₀) currents. Carisoprodol was significantly more efficacious at receptors incorporating the α 1 subunit compared to receptors expressing any other α subunit isoform. Potency was not affected by α subunit isoform, but was enhanced in β 1 vs. β 2 expressing receptors. Each data point represents the mean \pm S.E.M. from n cells. Significant differences compared to α 1 β 2 γ 2 receptors are denoted by *, p< 0.05.

Receptor Configuration	EC₅₀ (μM)	Efficacy (%)	n
α1β2	87.4 ± 16.4	346.7 ± 67.6	3
α1β2γ2	88.2 ± 19.9	474.7 ± 53.5	7
α2β2γ2	64.9 ± 19.1	198.5 ± 9.5*	6
α3β2γ2	63.7 ± 16.5	242.7 ± 35.1*	4
α4β2γ2	72.3 ± 10.1	240.0 ± 16.4*	11
α5β2γ2	90.8 ± 14.7	247.6 ± 23.0*	5
α6β2γ2	79.8 ± 13.8	232.6 ± 26.9*	9
α1β1γ2	33.1 ± 4.0*	225.6 ± 14.6*	6

TABLE II-3

Table II-4. Comparison of carisoprodol effects on δ subunit-containing extrasynaptic receptors with synaptic GABA_A receptors. Carisoprodol direct gating EC₅₀ was determined relative to maximum current elicited by carisoprodol and efficacy is normalized to maximum GABA gated currents. EC₅₀ for allosteric modulation was calculated in the presence of GABA (EC₂₀) and efficacy was calculated as maximum current elicited by carisoprodol plus GABA (EC₂₀) normalized to GABA (EC₂₀) currents. Carisoprodol was supra-efficacious at δ containing extrasynaptic GABA_A receptors for direct gating effects and significantly more efficacious at synaptic receptors incorporating the β 2 subunit for allosteric modulatory effects. Each data point represents the mean \pm S.E.M. from n cells. Significant differences are denoted by #, p< 0.01 (compared to synaptic receptors); ^, p< 0.05 (compared to α 1 extrasynaptic receptor) and **, p< 0.01 (compared to rest of the receptors studied).

Receptor Configuration	GABA EC ₅₀		CSP gating			CSP modulation		
	μM	n	Efficacy	EC ₅₀	n	Efficacy	EC ₅₀	n
α1β2	1.15 ± 0.08	4	31.5 ± 7.87	400 ± 37.4	6	442.2 ± 27.7 **	120 ± 10.5	8
α1β3	0.67 ± 0.06	6	37.1 ± 3.28	390 ± 48.3	9	234.5 ± 18.9	135 ± 12.8	4
α1β3γ2	04.3 ± 0.68	6	44.8 ± 3.23	436 ± 73.2	4	200.4 ± 09.5	131 ± 12.4	7
α1β3δ	1.37 ± 0.09	9	119.8 ± 4.7#	718 ± 36.2	9	241.5 ± 25.1	109 ± 11.4	8
α4β3δ	0.051 ± 0.03	6	135.6 ± 7.2#	242 ± 38.2^	5	181.5 ± 4.02	92.2 ± 15.1	5

TABLE II-4

Figure II-1. Influence of GABA_AR subunit isoforms on direct activation by carisoprodol. **A**, representative traces demonstrating carisoprodol activates human $\alpha 1\beta 2\gamma 2$, $\alpha 3\beta 2\gamma 2$, $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 2$ GABA_ARs in a concentration-dependent manner. Carisoprodol-activated currents are presented relative to the maximum current elicited by GABA (100 μ M for $\alpha 1\beta 2$ and 1 mM GABA for $\alpha 1\beta 2\gamma 2$, $\alpha 3\beta 2\gamma 2$ and $\alpha 1\beta 1\gamma 2$). At millimolar concentrations, rebound currents were observed upon termination of drug application. **B**, concentration-response curves for carisoprodol-mediated currents for human $\alpha 1$ -, $\alpha 2$ -, $\alpha 3$ -, $\alpha 4$ -, $\alpha 5$ -, and $\alpha 6\beta 2\gamma 2$ GABA_ARs. $\alpha 3$ - subunit expressing receptors displayed significantly less potency and efficacy to carisoprodol. Initial peak currents and not rebound currents elicited by carisoprodol were normalized to maximum GABA mediated currents. **C**, concentration-response curves for carisoprodol-mediated currents recorded from $\alpha 1\beta 2\gamma 2$ and $\alpha 1\beta 1\gamma 2$ GABA_ARs. Carisoprodol was significantly more efficacious at $\beta 1$ -containing receptors. **D**, concentration-response curves for carisoprodol-mediated currents recorded from $\alpha 1\beta 2$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs. There were no significant differences between the two configurations at each concentration tested ($p > 0.05$). Each data point represents the mean \pm S.E.M. of minimum of eight cells. *, $p < 0.05$; **, $p < 0.01$.

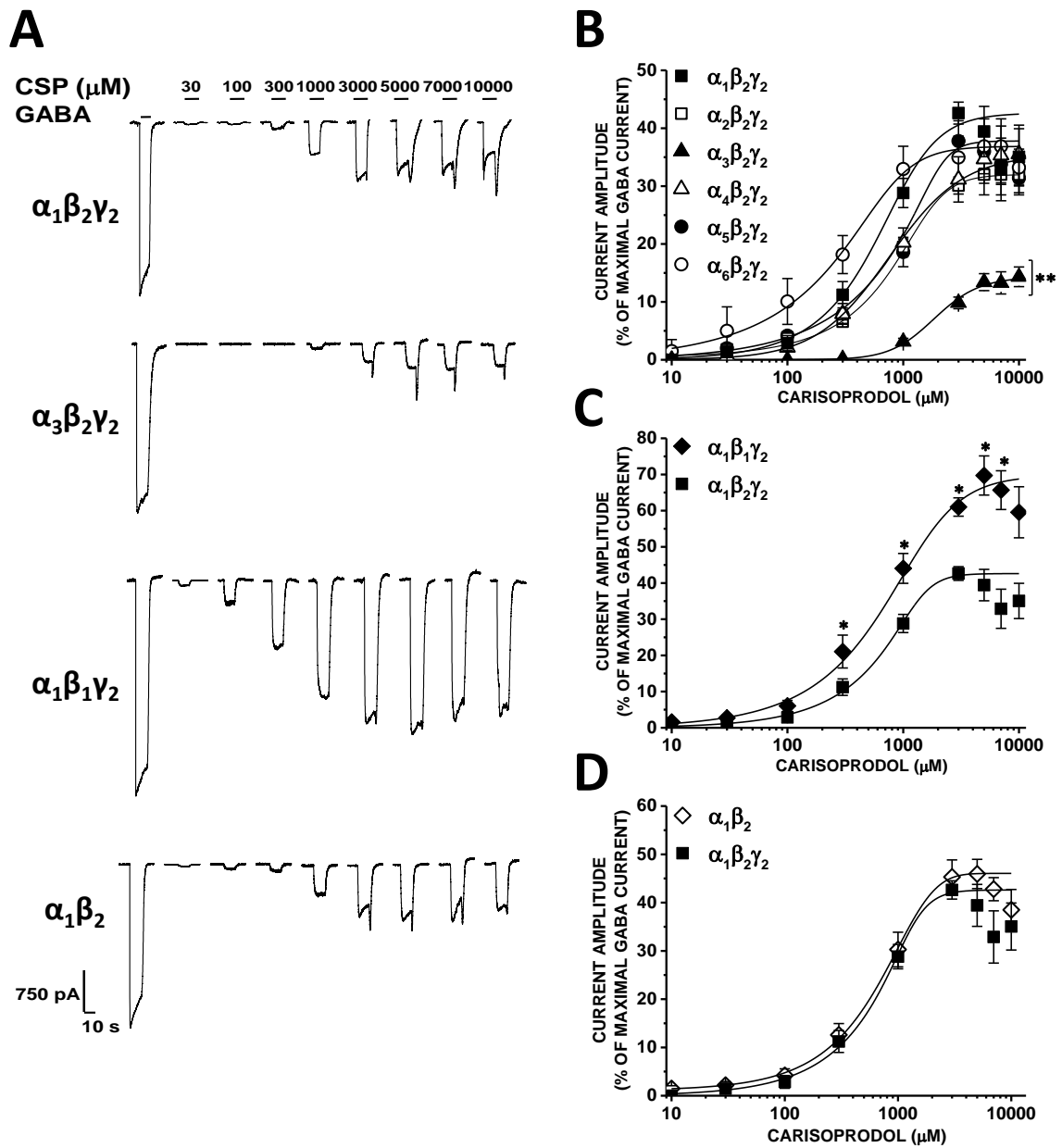


FIGURE II-1

Figure II-2. Influence of GABA_AR subunit isoforms on allosteric modulation by carisoprodol. **A**, representative traces demonstrating the potentiation of GABA-gated (EC₂₀) currents from human $\alpha 1\beta 2\gamma 2$, $\alpha 3\beta 2\gamma 2$, $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 2$ GABA_ARs by carisoprodol. **B**, concentration-response curves for the allosteric modulation of GABA-gated currents from human $\alpha 1$ -, $\alpha 2$ -, $\alpha 3$ -, $\alpha 4$ -, $\alpha 5$ -, and $\alpha 6\beta 2\gamma 2$ GABA_ARs by carisoprodol. Peak current elicited by carisoprodol plus GABA (EC₂₀) normalized to GABA (EC₂₀) mediated currents. Carisoprodol was significantly more efficacious at $\alpha 1$ -containing receptors. **C**, concentration-response curves for the allosteric modulation of GABA-gated currents recorded from $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs by carisoprodol. Carisoprodol was significantly more efficacious at $\beta 2$ -containing receptors. **D**, concentration-response curves for the allosteric modulation of GABA-gated currents recorded from $\alpha 1\beta 2$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs by carisoprodol. There were no significant differences between the two configurations at each concentration tested ($p > 0.05$). **E**, GABA concentration response in human $\alpha 1\beta 2\gamma 2$ GABA_A receptors in the absence (open square) or presence (filled square) of 100 μ M carisoprodol. Carisoprodol significantly decreased the GABA EC₅₀ (from 35.5 ± 0.64 to 8.2 ± 0.26 μ M ($n = 6$ and 4 , respectively)). The average carisoprodol direct gating effect at 100 μ M (2.8 % of maximal GABA-gated current) was subtracted from the summary data. Each data point represents the mean \pm S.E.M. of minimum of three cells. *, $p < 0.05$, ***, $p < 0.001$.

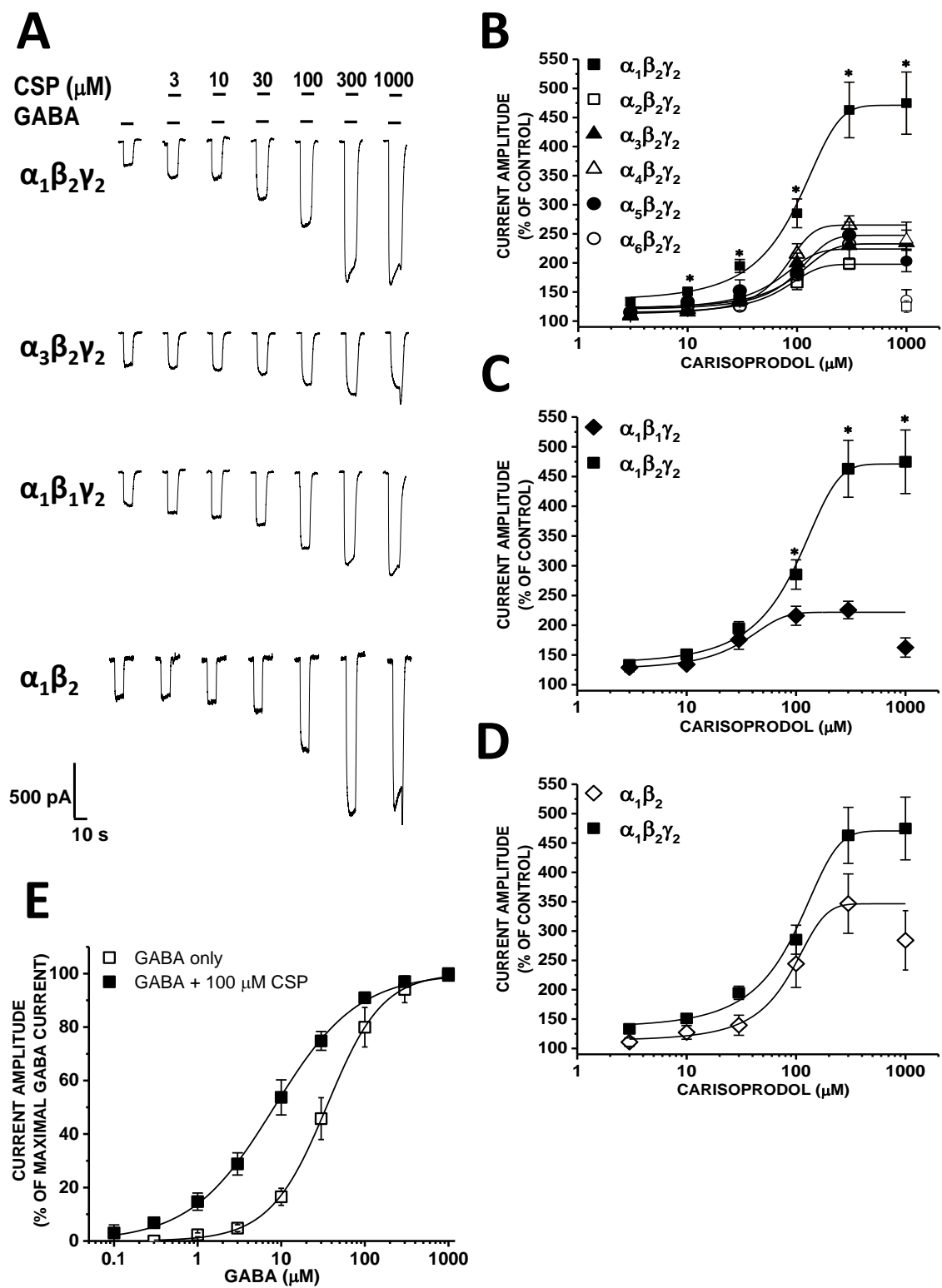


FIGURE II-2

Figure II-3. Direct gating action of carisoprodol on extrasynaptic GABA_A receptors.

A, representative traces demonstrating carisoprodol (CSP, 1 or 3 mM) activation of $\alpha 1\beta 2$, $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ compared to δ - expressing extrasynaptic $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ GABA_ARs. GABA concentrations are saturating at each receptor. At 3 mM and above, the amplitude of CSP-gated current in $\alpha 4\beta 3\delta$ decreases significantly, followed by a rebound current. **B**, concentration-response curves for carisoprodol-mediated currents in $\alpha 1\beta 3$, $\alpha 1\beta 3\gamma 2$, $\alpha 1\beta 2$ and extrasynaptic $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ GABA_ARs. The δ subunit-containing extrasynaptic GABA_ARs showed highest efficacy for carisoprodol. **C**, effect of the super agonist THIP in $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ GABA_ARs, compared to GABA. **D**, summary data demonstrating carisoprodol's efficacy on δ - expressing extrasynaptic $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ GABA_ARs compared to THIP. Carisoprodol is approximately 90% and 88% efficacious as THIP on $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ GABA_ARs, respectively. Each data point represents the mean \pm S.E.M. of minimum of four cells. #, $p < 0.01$.

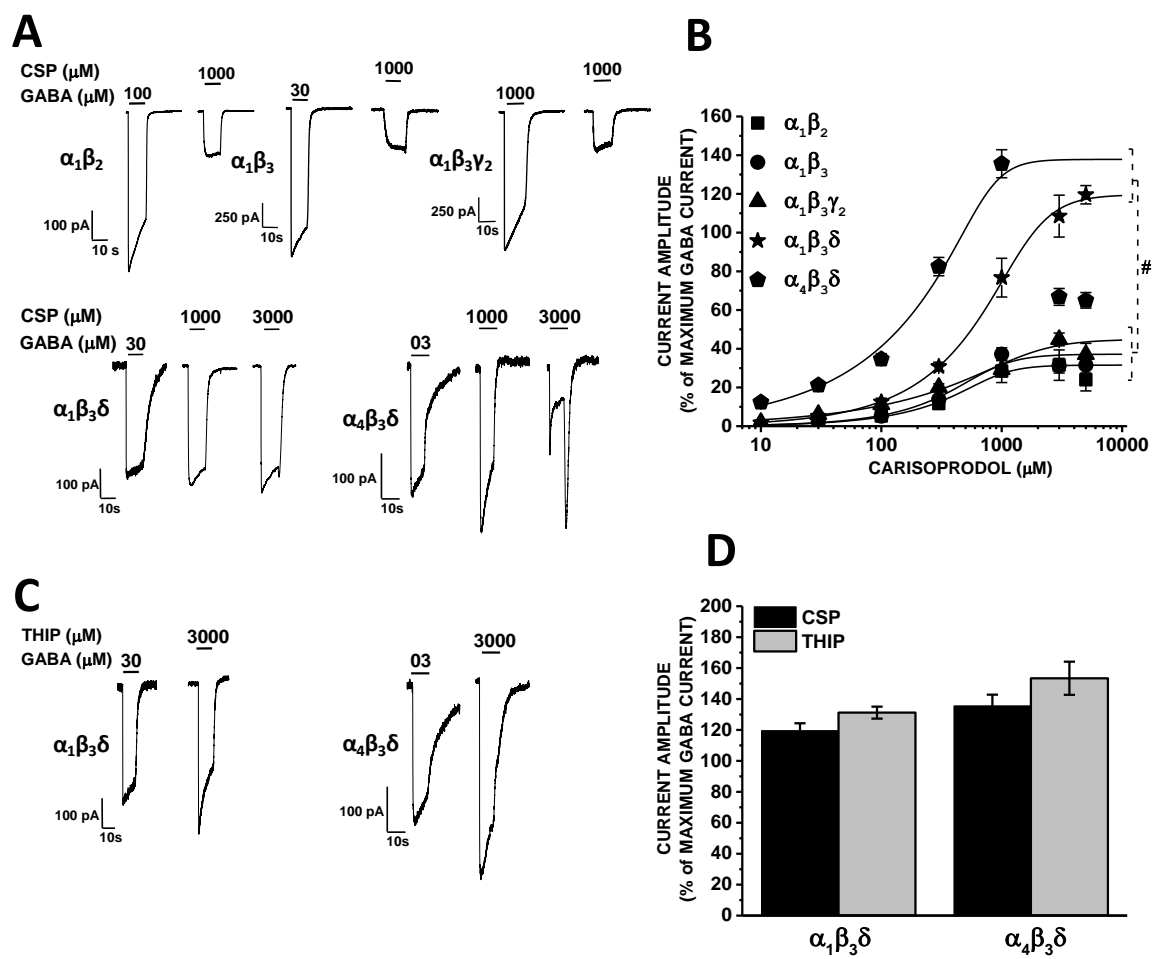
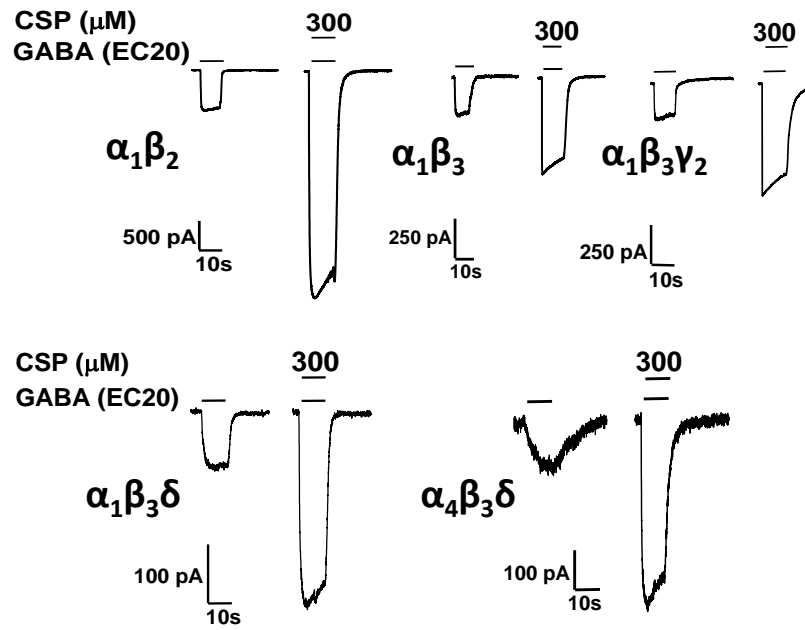


FIGURE II-3

Figure II-4. Allosteric modulation of extrasynaptic GABA_A receptors by carisoprodol. **A**, representative traces demonstrating the potentiation by carisoprodol (300 μ M) of GABA-gated (EC₂₀) currents in α 1 β 3, α 1 β 3 γ 2 and α 1 β 2 compared to δ -expressing extrasynaptic α 1 β 3 δ and α 4 β 3 δ GABA_ARs. **B**, concentration-response curves showing mean allosteric carisoprodol modulation of GABA-gated currents in α 1 β 3, α 1 β 3 γ 2, α 1 β 2 and extrasynaptic α 1 β 3 δ and α 4 β 3 δ GABA_ARs. GABA_ARs containing β 2 subunits showed highest efficacy for carisoprodol. Each data point represents the mean \pm S.E.M. of a minimum of four cells. *, $p < 0.05$; **, $p < 0.01$.

A



B

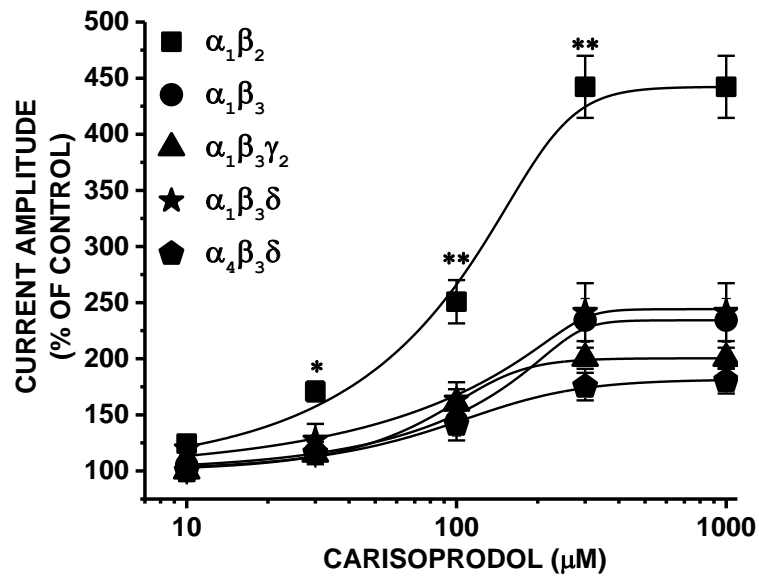


FIGURE II-4

Figure II-5. Potentiation of saturating GABA current by sub-gating concentration of carisoprodol in extrasynaptic GABA_A receptors. **A**, representative traces demonstrating the potentiation of saturating GABA currents by carisoprodol and pentobarbital in extrasynaptic $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ GABA_ARs. **B**, bar graph summarizing carisoprodol's significant potentiation of maximal GABA currents in extrasynaptic $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ GABA_ARs, as seen with pentobarbital. This effect was not seen in $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ GABA_ARs. Each data point represents the mean \pm S.E.M. of a minimum of four cells. **, $p < 0.01$.

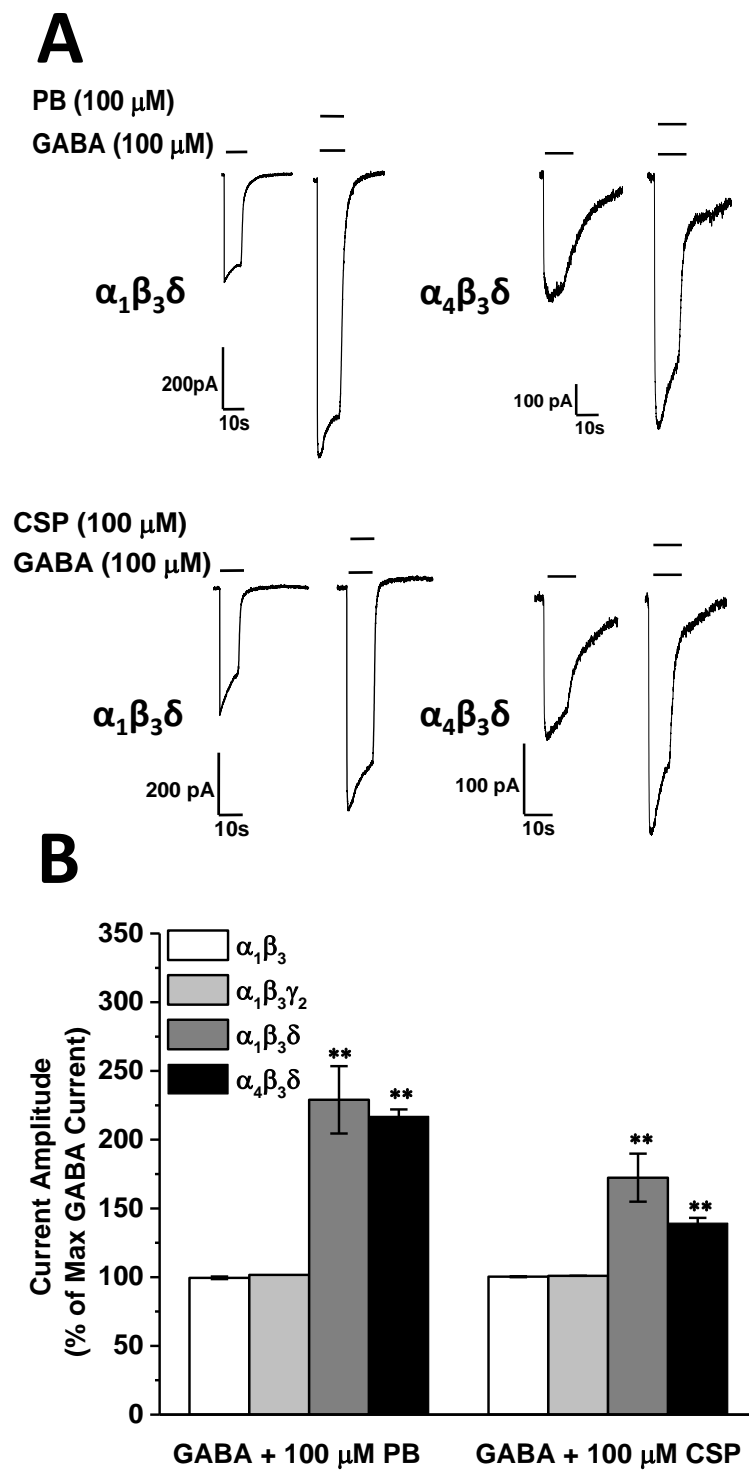


FIGURE II-5

Figure II-6. Voltage-dependent effect of carisoprodol actions on GABA_A receptors. A, C and E, representative traces from $\alpha 1\beta 2\gamma 2$ GABA_ARs transfected HEK293 cells clamped at -60 mV and +60 mV for carisoprodol's direct gating, blocking and allosteric modulatory effects. **B, D and F,** bar graph summarizing carisoprodol direct gating, blocking and allosteric modulatory actions, respectively, at -60 mV and +60 mV. Carisoprodol direct gating and blocking effects are independent of voltage. However, allosteric potentiation of GABA currents by carisoprodol (100 μ M) was significantly reduced compared to the level of potentiation observed at -60 mV. Each data point represents the mean \pm S.E.M. of minimum of four cells. *, $p < 0.01$.

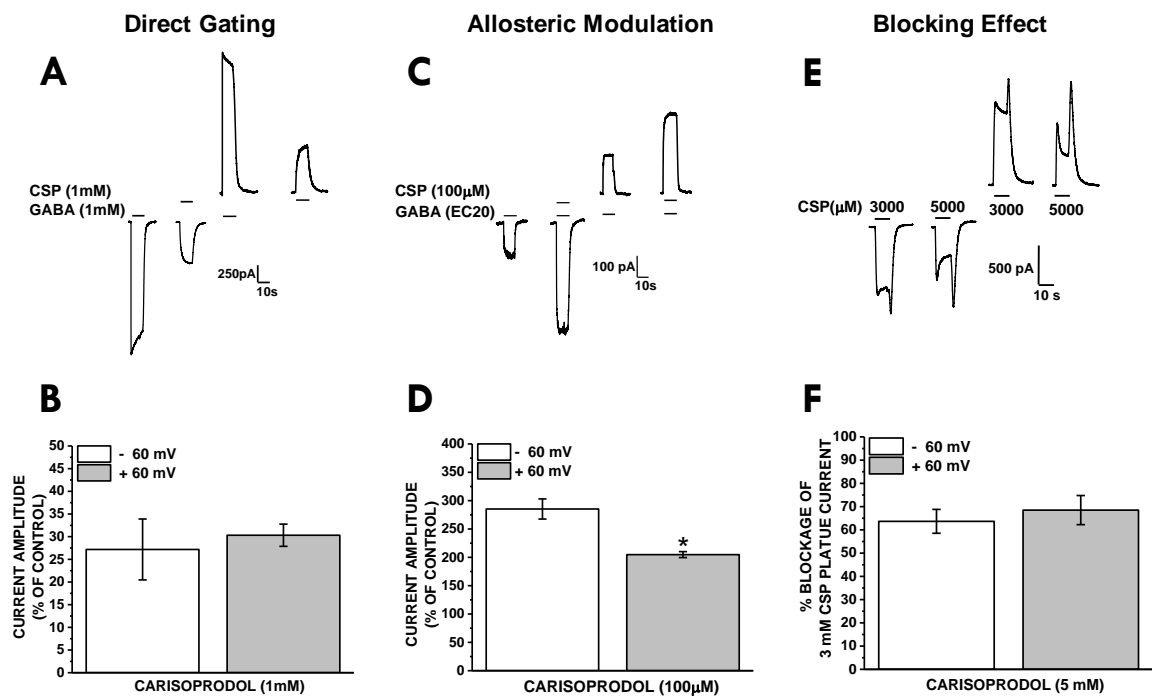


FIGURE II-6

AUTHORSHIP CONTRIBUTIONS

Participated in research design: **Kumar, Gonzalez, and Dillon**

Conducted experiments: **Kumar and Gonzalez**

Performed data analysis: **Kumar, Gonzalez, and Dillon**

Wrote or contributed to writing of the manuscript: **Kumar, Gonzalez, and Dillon**

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Footnotes

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CHAPTER III

A SINGLE AMINO ACID RESIDUE AT TRANSMEMBRANE 4 OF THE ALPHA 1 SUBUNIT INFLUENCES CARISOPRODOL DIRECT GATING EFFICACY AT GABA_A RECEPTORS

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ABSTRACT

The muscle relaxant carisoprodol (CSP, trade name Soma) has recently been controlled at the federal level as a Schedule IV drug due to its high abuse potential and consequences of misuse, such as withdrawal syndrome, delusions, seizures and even death. Recent work has shown that carisoprodol can directly gate and allosterically modulate the GABA_A receptor. These actions are subunit-dependent; compared to other GABA_A receptors, carisoprodol has nominal direct gating effects in $\alpha 3\beta 2\gamma 2$ receptors. Here, using site-directed-mutagenesis and whole cell patch clamp electrophysiology in transiently transfected HEK293 cells, we examined the role of GABA_A receptor α subunit transmembrane domain 4 (TM4) amino acids in direct gating and allosteric modulatory actions of carisoprodol. Mutation of $\alpha 3$ valine at position 440 to leucine (present in the equivalent position in the $\alpha 1$ subunit) increased the direct gating potency and efficacy of carisoprodol significantly, without affecting allosteric modulatory effects. The corresponding reverse mutation, $\alpha 1$ (L415V), decreased carisoprodol direct gating potency and efficacy. Subsequent assessment of a series of amino acids at $\alpha 1$ (415) on carisoprodol efficacy showed the following rank order: (L = I = T = R > Y > W = C = V > G > S). We conclude $\alpha 1$ (415) of TM4 is involved in the direct gating, but not in allosteric modulatory, actions of carisoprodol. Also, orientation of alkyl or hydroxyl groups at this position influence direct gating effects. These findings support the likelihood that direct gating and allosteric modulatory effects of carisoprodol are mediated via distinct binding sites.

INTRODUCTION

Carisoprodol (*N*-isopropyl meprobamate, CSP) is a centrally acting muscle relaxant frequently prescribed for skeleton muscle pain (Luo et al., 2004; Toth and Urtis, 2004). In recent years, misuse and abuse of carisoprodol has become a significant problem. Carisoprodol abuse causes psychomotor impairment and severe withdrawal that may predispose to seizures and death (Bramness et al., 2004; Fass, 2010; Reeves et al., 2012; Zacny and Gutierrez, 2011; Zacny et al., 2011). Tolerance to carisoprodol develops relatively fast, facilitating the problems associated with withdrawal (Gatch et al., 2012; Reeves and Burke, 2010). As per the National Survey on Drug Use and Health in 2009, an estimated 2.9 million people in US admitted that they had consumed carisoprodol for non-medical purpose (see ref: Drugs and chemical of concerns). Indeed, considering its alarming abuse rate, effective January of 2012, carisoprodol was scheduled at the federal level (see ref: Schedule of controlled substances). Considering the danger posed by this drug, there is urgent need for improved treatment for carisoprodol toxicity, and a novel drug with less abuse potential.

Until recently, it was widely accepted that the sedative and muscle relaxing effects of carisoprodol were predominantly due to its primary metabolite, meprobamate (Bramness et al., 2004). More recent work has shown that carisoprodol itself allosterically modulates, directly activates and blocks γ -Aminobutyric acid, type A (GABA_A) receptors in a concentration-dependent manner (Gonzalez et al., 2009). In vivo studies also support the fact that carisoprodol itself has significant CNS effects due to interaction with GABA_A receptors (Gonzalez et al., 2009).

GABA_A receptors are member of the cys-loop family of ligand-gated ion channels; they are hetero-pentameric Cl⁻ channels and play a critical role in mediating fast inhibition in the brain (Corringer et al., 2012; Sigel and Steinmann, 2012). Multiple GABA_A receptor subunits and corresponding isoforms have been identified, including α (1-6), β (1-3), γ (1-3), ρ , δ , ϵ and θ (Olsen and Sieghart, 2008). Each subunit is composed of a large extracellular N terminus, four transmembrane helices (TM1–TM4), an extracellular TM2–TM3 loop, a large TM3–TM4 intracellular loop, and an extracellular C terminus (Cockcroft et al., 1995). The TM2 domains form the pore of the channel (Miyazawa et al., 2003; Xu and Akabas, 1996) (Fig. 1). In addition to the GABA binding site, GABA_A receptors have binding sites for several clinically important drugs, including anxiolytics, sedative-hypnotics, muscle relaxants, and anesthetics. The GABA binding site is located at the interface of the $\alpha 1$ and $\beta 2$ subunits, and benzodiazepines bind at the interface of $\alpha 1$ - $\gamma 2$ subunits in the extracellular region (Newell and Czajkowski, 2003; Sigel and Steinmann, 2012) (Fig 1A). Barbiturate and general anesthetic (propofol, etomidate) binding sites are believed to be positioned in the water accessible region located between the TM helices of the receptor (Bali and Akabas, 2004; Siegwart et al., 2002; Zeller et al., 2007a). Carisoprodol actions are not mediated via reported sites of action for benzodiazepines or barbiturates (Gonzalez et al., 2009). While the general anesthetics propofol and etomidate allosterically modulate and directly gate GABA_A receptors through a single site of action (Siegwart et al., 2002; Stewart et al., 2013), distinct GABA_A receptors sites confer these properties to neurosteroids (Hosie et al., 2006).

Although not definitive, work to date suggests carisoprodol may mediate its effects via distinct sites of action (Gonzalez et al., 2009).

Our recent studies with carisoprodol on GABA_A receptors have shown the allosteric modulatory and direct gating properties of carisoprodol are subunit-dependent (Kumar et al., 2015). Allosteric modulatory actions of carisoprodol are most efficacious at receptors incorporating the $\alpha 1$ subunit, whereas $\alpha 3$ -expressing receptors show minimal direct gating effects. Characteristics of carisoprodol effects are consistent with it interacting at the transmembrane domains (Hosie et al., 2006). Aligned amino acid sequences of human α subunit isoforms ($\alpha 1$ -6) revealed that TM1, TM2 and TM3 are fully conserved in all α subunit isoforms. The TM4 region of α subunit isoforms is also largely conserved; however, I419, I423 and V440 residues of $\alpha 3$ differ compared to all other α subunit isoforms (Fig. 1C) (Barnard et al., 1998; Bergmann et al., 2013). We thus explored the extent to which these residues may contribute to the ability of carisoprodol to directly gate and allosterically modulate GABA_A receptors. We have identified L415 at TM4 of the $\alpha 1$ subunit as being critically involved in direct gating actions of carisoprodol.

MATERIAL AND METHODS

Plasmids and site-directed-mutagenesis

Human cDNA plasmids encoding $\alpha 1$, $\alpha 3$, $\beta 2$ and $\gamma 2$ GABA_A receptor subunits were used in the present study. Individual and combined mutations in $\alpha 1$ and $\alpha 3$ cDNA plasmids were created using Stratagene's Quik Change II ® site-directed-mutagenesis kit (Agilent Technologies; La Jolla, CA) and were sequenced to confirm mutations at West Virginia University's Genomics Core Facility.

Chemicals and solutions

Carisoprodol, pentobarbital, salts and buffers were purchased from Sigma Aldrich (St. Louis, MO), GABA was obtained from Acros Organics (New Jersey, US). Pentobarbital and GABA stock solutions (500 mM) were prepared in deionized water. Carisoprodol stock solution (1 M) was made in DMSO. All stock solutions were stored at -20° C. On the day of experiment, fresh working drug concentrations were prepared from stock solution by dissolving in physiological buffer solution (below).

Cell Culture and Transfection

Human embryonic kidney 293 (HEK293) cells were transfected with human cDNA encoding desired GABA_A receptor subunits. To obtain $\alpha\beta\gamma 2$ GABA_A receptors, HEK293 cells were transfected with human GABA_A $\alpha 1/3$ mutant or wild type ; human $\beta 2$; and human $\gamma 2s$ (short isoform) subunit cDNA in a 1:1:5 (0.3 μ g : 0.3 μ g : 1.5 μ g) ratio using poly jet DNA

in vitro transfection reagent (SigmaGen Laboratories, MD) and used for recording 24-48 h later. The $\gamma 2s$ subunit will be referred to as $\gamma 2$ from this point forward. Human GABA_A $\alpha 1$ subunit cDNA was generously provided by Neil Harrison (Columbia University Medical Center, New York). Cells were plated on glass coverslips coated with poly-L-lysine in 35-mm culture dishes and were incubated and maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂.

Whole-cell patch clamp electrophysiology

All experiments were conducted at room temperature (22-25°C) with the membrane potential clamped at -60 mV. Patch pipettes of borosilicate glass (1B150F; World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC; Sutter Instrument Company, Novato, CA) to a tip resistance of 4–6 M Ω . Patch pipettes were filled with a solution consisting of 140 mM CsCl, 10 mM EGTA-Na⁺, 10 mM HEPES-Na⁺, and 4 mM Mg²⁺-ATP, pH 7.2. Coverslips containing cultured cells were placed in the recording chamber on the stage of an inverted light microscope and superfused continuously with an external solution consisting of 125 mM NaCl, 20 mM HEPES, 3 mM CaCl₂, 5.5 mM KCl, 0.8 mM MgCl₂, and 10 mM glucose, pH 7.4. Agonist-induced Cl⁻ currents were obtained with an Axopatch 200B amplifier with a rate of 50 samples per second (Molecular Devices, Sunnyvale, CA) equipped with a CV-203BU head stage. Currents were low-pass filtered at 5 kHz, monitored simultaneously on an oscilloscope and a chart recorder (Gould TA240;

Gould Instrument Systems Inc., Cleveland, OH), and stored on a computer using an on-line data acquisition system (pCLAMP 6.0; Axon Instruments) for subsequent off-line analysis.

Experimental Protocol

GABA (with or without carisoprodol) or carisoprodol was prepared in external saline solution from stock solutions and applied to each cell by gravity flow using a Y-shaped tube positioned adjacent to the cell. Recordings were obtained from transfected cells only after establishing that two consecutive GABA EC₂₀-activated currents varied in amplitude by no more than $\pm 10\%$. For studies investigating direct activation, carisoprodol-mediated currents were normalized to currents elicited by saturating GABA concentrations. Modulatory effects of carisoprodol on GABA-gated currents were assessed using an EC₂₀ gating concentration of GABA as the control (individually determined for each mutant and wild type receptor studied). This gating concentration was selected to ensure there was a sufficient range to observe the full allosteric potential of carisoprodol. At the initiation of each recorded cell, it was confirmed that gating concentration was approximately the EC₂₀ (range of EC₁₅ to EC₂₅ accepted for an individual cell). In recordings displaying inhibition followed by a rebound current after termination of carisoprodol or carisoprodol plus GABA application (Gonzalez et al., 2009), the maximal current amplitude achieved during active ligand application was taken as the peak current.

Data Analysis

Concentration-response profiles for the positive modulatory actions of carisoprodol were generated (Origin 9.1; OriginLab Corp., Northampton, MA) using the equation $I/I_{\max} = [\text{carisoprodol}]^n / ([\text{carisoprodol}]^n + \text{EC}_{50}^n)$, where I is the normalized current amplitude at a given concentration of carisoprodol, I_{\max} is the maximum current induced by carisoprodol, EC_{50} is the half-maximal effective concentration of carisoprodol, and n is the Hill coefficient. For concentration-response curves illustrating allosteric actions, a correction was applied to subtract direct gating effects. In some cases, the blocking actions of carisoprodol became notable at high concentrations; in these instances, curves were fitted to the data point corresponding to peak effect, and the curve was extrapolated. All data are presented as mean values \pm S.E. Statistical significance between control and test conditions was determined using Student's t -test (paired or unpaired) and one-way analysis of variance. Tukey-Kramer *post hoc* test for multiple comparisons was performed as needed. Correlation assessments were performed using linear fit in origin 9.1.

RESULTS

Functional characterization of $\alpha 3$ and $\alpha 1$ TM4 mutant GABA_A receptors

For this study, an extensive series of mutations (single point or 2-3 residues) in $\alpha 1$ and $\alpha 3$ subunits were evaluated. In all cases, the mutant subunit was expressed with wild type $\beta 2$ and $\gamma 2$ subunits, and GABA concentration-response profiles were generated to assess overall receptor function and to establish gating concentrations for allosteric studies. GABA EC₅₀ for wild type $\alpha 1$ and $\alpha 3$ receptors were both approximately 35 μ M (Tables 1 and 2). In general, shifts in GABA EC₅₀ were modest. Mutations in $\alpha 3$ subunits caused a leftward shift in the GABA concentration-response curve of 1.9- to 4.6-fold (Table 1). Similarly, mutations in the $\alpha 1$ subunit had either insignificant or modest effects on GABA EC₅₀, with the maximal effect being a 2.5-fold increase in GABA EC₅₀ relative to wild type $\alpha 1$ receptors (Table 2). Thus the mutations had minimal effects on fundamental receptor gating.

Mutation of $\alpha 3$ TM4 amino acids to corresponding $\alpha 1$ amino acids increased direct gating effect of carisoprodol but not allosteric modulatory actions

Consistent with our previous report (Kumar et al., 2015), the ability of carisoprodol to directly gate $\alpha 3\beta 2\gamma 2$ receptors was significantly attenuated compared to GABA_A receptors expressing the $\alpha 1$ subunit (Fig. 2B, Tables 1, 2). Maximal current amplitudes generated by 3 mM carisoprodol were 41.8 ± 2.4 % and 8.5 ± 1.1 % of that generated by saturating GABA in $\alpha 1\beta 2\gamma 2$ and $\alpha 3\beta 2\gamma 2$ receptors, respectively. We thus assessed the potential involvement of three amino acids we identified in TM4 that were unique to the $\alpha 3$ subunit in this

attenuated direct gating effect. Residues were mutated to the amino acid found at the equivalent position in the $\alpha 1$ subunit, either alone or in combination. As shown in Figure 2, all $\alpha 3$ mutants resulted in a gain-of-function effect, significantly increasing direct gating currents such that current amplitudes in response to 3 mM carisoprodol were not significantly different from that obtained in wild type $\alpha 1\beta 2\gamma 2$ receptors (summary values in Table 1).

As carisoprodol also has a diminished allosteric modulatory effect in $\alpha 3\beta 2\gamma 2$ receptors compared to $\alpha 1\beta 2\gamma 2$ receptors (Kumar et al., 2015), we also assessed the extent to which these mutations might increase sensitivity to the allosteric actions of carisoprodol. Interestingly, allosteric modulatory effects of carisoprodol were not affected by mutations. Whereas carisoprodol enhanced GABA EC₂₀ currents in all mutated receptors, the magnitude of the effect for each mutant was not significantly different from that observed in $\alpha 3\beta 2\gamma 2$ receptors, and the allosteric potentiation fell far short of that obtained in $\alpha 1\beta 2\gamma 2$ receptors (Fig. 2D and Table 1).

A single mutation of $\alpha 1$ TM4 L415 amino acid to corresponding $\alpha 3$ V440 amino acid decreased direct gating effect of carisoprodol.

To confirm the involvement of the identified α subunit TM4 residues in direct gating action of carisoprodol, we mutated $\alpha 1$ TM4 amino acids to the corresponding $\alpha 3$ TM4 three non-identical amino acids (L394I, A398I, and L415V) (Fig. 1) in all possible combinations of single, double or triple mutation, and assessed direct gating by carisoprodol. Out of seven mutations generated, each of those containing the L415V mutation caused a

significant loss of carisoprodol (3 mM) direct gating effect compared to wild type $\alpha 1$ -expressing receptors, whereas carisoprodol's effects in those receptors not incorporating the L415V mutation were not significantly different from wild type (Fig 3A, 3B and Table 2). These data indicate the leucine residue at position 415 in the $\alpha 1$ subunit has a key role in the direct gating action of carisoprodol.

Amino acid residue at $\alpha 1(415)$ subunit influences carisoprodol direct gating efficacy.

In an attempt to gain additional insight into physicochemical determinants that influence carisoprodol direct gating capability at the $\alpha 1(415)$ position, we generated and assessed the following series of mutations: $\alpha 1(L415S)$, $\alpha 1(L415G)$, $\alpha 1(L415T)$ $\alpha 1(L415Y)$ $\alpha 1(L415W)$, $\alpha 1(L415I)$, $\alpha 1(L415C)$ and $\alpha 1(L415R)$. These residues provide a range of amino acid side chain properties, including volume, polarity and hydrophathy. We tested each mutant receptor for carisoprodol direct gating potency and efficacy. Similar to valine substitution at $\alpha 1$ (L415) position, L415S, L415G, L415W and L415C all decreased maximal gating efficacy (to 5.5 ± 1.2 , 9.1 ± 1.6 , 17.8 ± 4.1 , and 18.0 ± 1.6 % of saturating GABA current, respectively). Potency to carisoprodol was generally unaffected, with the exception that the L415W mutant induced a three-fold rightward shift in EC_{50} (Fig 4 A, B, C and Table 2). The presence of T, Y, I or R had no effect on either carisoprodol efficacy or potency. Correlation analysis showed positive and negative correlations of amino acid volume and polarity at the 415 position with carisoprodol direct gating efficacy (Fig. 5). These data demonstrate the nature of the amino acid side chain at $\alpha 1(415)$ position is critical for the

direct gating effect of carisoprodol. In addition, an increase in GABA EC₅₀ correlated negatively with carisoprodol efficacy (Fig. 5F).

The $\alpha 1$ (L415S) mutation does not affect allosteric modulation by carisoprodol or direct activation by pentobarbital

To further assess the extent to which the $\alpha 1$ (L415) residue may be differentially involved in direct gating action allosteric modulatory effects of carisoprodol, we tested if the L415S mutation, which effectively obliterated the ability of carisoprodol to directly gate the receptor (Fig. 4), had an effect on allosteric potentiation. In $\alpha 1$ (L415S) $\beta\gamma 2$ receptors, carisoprodol potentiation of GABA EC₂₀ currents differed in neither maximum potentiation nor potency when compared to wild type receptors ($439.45 \pm 49.4\%$ potentiation and EC₅₀ of $89.5 \pm 15 \mu\text{M}$, $n = 7$, in $\alpha 1$ (L415S) $\beta\gamma 2$ receptors compared to $474.75 \pm 53.4\%$ and $102.2 \pm 16 \mu\text{M}$, $n = 5$ in wild type receptors, Fig. 6A, B). As a further assessment of the specificity of the L415S mutation, we evaluated if it had any effect on direct gating by pentobarbital. Direct gating by 1 mM pentobarbital was not significantly different in $\alpha 1$ (L415S) $\beta\gamma 2$ receptors compared to wild type receptors (current amplitude in comparison to saturating GABA was $70.2 \pm 4.2\%$; $n = 5$ and $84.1 \pm 6.4\%$; $n = 7$ in mutant and wild type receptors, respectively (Fig 6. C and D). Thus effects of the L415S mutation are not due to non-specific effects on the ability of direct-gating ligands to activate the channel.

DISCUSSION

In a recent report (Kumar et al., 2015), we found that direct gating effects of the skeletal muscle relaxant carisoprodol were reduced in $\alpha 3\beta\gamma 2$ GABA_A receptors, compared to those expressing any other α subunit variant ($\alpha 1$ -2, $\alpha 4$ -6). Here, we identified α subunit TM4 residues, in particular $\alpha 1415$ (equivalent to $\alpha 3440$), that are critical for direct gating, but not allosteric modulatory, effects of carisoprodol (Fig 1). Mutation of the native $\alpha 3$ 440V residue to the L residue found in the $\alpha 1$ subunit (V440L) resulted in a significant enhancement of carisoprodol-gated current; the converse mutation (L415V), had the opposite effect (Fig 2 and 3). Subsequent evaluation of a series of mutations resulted in the following rank order effect on carisoprodol gating efficacy (L = I = T = R > Y > W = C = V > G > S), and correlation analysis demonstrated that both amino acid volume and polarity are important determinants of this position's effect on carisoprodol direct gating (Fig 5). The presence of a hydrophobic residue tended to correlate with enhanced carisoprodol gating, although this effect did not reach statistical significance. Interestingly, except for tryptophan (which caused a 3-fold increase in carisoprodol direct gating EC₅₀), none of the introduced mutants affected potency of carisoprodol's direct gating effect; the action was nearly exclusively an effect on efficacy.

Transmembrane 4 residues of the $\alpha 1$ subunit have been shown to be involved in allosteric modulatory effect of other GABA_A receptor ligands, such as neurosteroids and anesthetics (Hosie et al., 2006; Jenkins et al., 2002). Homology modeling has shown that

N407 and Y410 donate a hydrogen bond to the ketone group of THDOC, and contribute to the binding pocket of neurosteroids. Substitution of polar residues to hydrophobic amino acids at N407A and Y410F reduced THDOC potency significantly (Hosie et al., 2006). Indeed, L415 itself has been implicated in effects on anesthetic agents. In a tryptophan scanning study of TM4, it was found that introduction of tryptophan at position 415 (L415W) of the $\alpha 1$ subunit produced a significant decrease in the ability of the anesthetics halothane and chloroform to potentiate GABA-gated currents (Jenkins et al., 2002). It is possible TM domains forms an important allosteric modulatory site on GABA_A receptors. However, our results would seem to rule out the potential involvement of the TM4 domain 415 position, as the (L415S) mutation did not affect allosteric potentiation of GABA currents (Fig 6). These results are consistent with our contention that distinct sites exist for the allosteric modulatory and direct gating effects of carisoprodol.

Previous molecular and behavior studies of carisoprodol have shown characteristics of barbiturate-like effects. Both ligands directly gate, allosterically modulate, and inhibit the receptor (at high concentrations). More notably, in drug discrimination studies, the barbiturate pentobarbital substituted for the discriminative stimulus effects of carisoprodol in carisoprodol-trained rats. In addition, the barbiturate antagonist bemegride blocked the locomotor depression effect of carisoprodol in mice, and also antagonized carisoprodol-gated currents in HEK293 cells expressing GABA_A receptors (Gonzalez et al., 2009). These findings suggested that behavior and molecular action of carisoprodol may be mediated by a barbiturate-like mechanism of action on GABA_A receptors. However, in the present study

the $\alpha 1$ (L415S) mutation did not affect the ability of pentobarbital to directly gate GABA_A receptors. In addition, a rho receptor (wild type is insensitive to barbiturates) mutation that confers sensitivity to barbiturate did not confer sensitivity to carisoprodol (Gonzalez et al., 2009). Thus, although previous studies have shown barbiturate-like action of carisoprodol, collectively the data support distinct binding sites or functional domains for carisoprodol versus barbiturate direct gating effects in GABA_A receptors. The actions of neurosteroids are also comparable to carisoprodol. A threonine in position 236 of the $\alpha 1$ subunit (T236) has been shown to be involved in direct gating of neurosteroids, as mutation of this residue to I greatly attenuates sensitivity to the neurosteroid THDOC (Hosie et al., 2006). In separate experiments, we tested whether the $\alpha 1$ (T236I) mutation affected carisoprodol direct gating actions. We found this mutation did not produce any change in carisoprodol efficacy relative wild type receptors. Thus, the site of action for carisoprodol appears to also be distinct from that of neurosteroids.

Our subunit-dependent studies of carisoprodol have also demonstrated significant influence of the β subunit isoform in direct gating and allosteric modulatory effects of carisoprodol (Kumar et al., 2015). Receptors expressing the $\beta 1$ subunit showed highest efficacy for direct gating, whereas $\beta 2$ -containing receptors confer enhanced allosteric modulatory properties. The TM2 15' residue lies toward the extracellular aspect of the channel, and faces away from the channel pore; this residue in the $\beta 2$ subunit (N265) has been shown to be involved in alcohol and general anesthetics actions at GABA_A receptors (McCracken et al., 2010). Mutation of the $\beta 2$ asparagine to methionine (N265M) reduced

the direct gating currents of propofol, alphaxolone and etomidate, and decreased allosteric potentiation by etomidate, propofol, enflurane, isoflurane, butanol and octanols (Siegwart et al., 2003; Zeller et al., 2007b). Interestingly, TM2 of β subunit isoforms is highly conserved. An exception is the 15' residue, which is S in the $\beta 1$ subunit. In preliminary studies in our lab, mutation of the $\beta 1$ TM2 15' serine to the corresponding $\beta 2$ subunit residue (asparagine, N) decreased the direct gating efficacy of carisoprodol to 22.4 ± 4.4 for $\alpha 1\beta 1(S_{TM2}15'N)\gamma 2$ receptor significantly from 62.2 ± 1.8 for $\alpha 1\beta 1\gamma 2$ wild type receptors. Based on our model of the TM domains of the GABA_A receptor (Fig. 7), it is unlikely carisoprodol docks between the 265 residue of the $\beta 2$ subunit and 415 of the $\alpha 1$ subunit. The involvement of the TM2 15' position in a diverse array of ligands suggests its effects are more likely on transduction, as opposed to forming part of a binding site for multiple and diverse ligands. A complete understanding of the binding pocket for carisoprodol requires further exploration.

It is known that addictive drugs hijack the reward system by increasing dopamine levels in mesolimbic system (Luscher and Ungless, 2006). Dopaminergic neurons in the ventral tegmental area (VTA) express $\alpha 3$ -containing GABA_A receptors and the GABAergic interneurons in the VTA express $\alpha 1$ -containing receptors (Tan et al., 2010). Diazepam binding to the $\alpha 1$ -containing GABA_A receptors on GABAergic VTA neurons leads to a reduction of the activity of these cells, and thus reduced release of GABA, which results in a disinhibition of the dopaminergic VTA neurons (Rudolph and Knoflach, 2011). In turn, this results in an increase in dopamine release in the ventral striatum (Heikkinen et al., 2009). These studies verified the $\alpha 1$ GABA_A receptor subtype in the mesolimbic dopaminergic

system is involved in addiction. Further, behavior studies in mice with mutation of a residue key for benzodiazepine's effects have shown physiological effects of this agent are GABA_A receptor subunit-dependent. The $\alpha 1$ (H101R) knock-in mice showed absence of sedative effects of diazepam but anxiolytic effects were not affected (Rudolph et al., 1999). If the mutation was instead present in the $\alpha 2$ subunit $\alpha 2$ (H101R), the anxiolytic action of diazepam was absent, its myorelaxant actions (which are observed at higher doses than anxiolytic effects) were reduced, but its sedative action was intact (Low et al., 2000). In $\alpha 3$ (H126R) and $\alpha 5$ (H105R) mice, the myorelaxant action of diazepam was reduced without altering sedative and anxiolytic actions (Crestani et al., 2001). Moreover, the development of tolerance to the sedative action of benzodiazepines has been linked to $\alpha 5$ -expressing GABA_A receptors (van Rijnsoever et al., 2004).

The goal of our present study was to identify key amino acid residues that are involved in actions of carisoprodol at GABA_A receptors. We found that $\alpha 1$ (L415) of transmembrane domain 4 of the GABA_A receptor is involved in direct gating action of carisoprodol, and identified physicochemical traits that are important for this effect. Mutation of this residue did not impact allosteric modulatory effects of carisoprodol, and it also had no effect on the ability of the barbiturate pentobarbital to directly gate the receptor. These results are consistent with our contention that carisoprodol mediates these two actions through distinct, likely novel, sites on the GABA_A receptor.

Table III-1. Influence of GABA_A receptor $\alpha 3$ TM4 amino acids mutation to corresponding $\alpha 1$ amino acids on GABA EC₅₀ values, carisoprodol direct activation and allosteric modulation. Carisoprodol direct gating activation at 3mM is normalized to saturated GABA current, whereas carisoprodol modulation effect to potentiate GABA-gated current is normalized to GABA EC₂₀ current. Each data point represents the mean \pm S.E.M of n cells. *, p < 0.05; **, p < 0.01 relative to wild type $\alpha 3\beta 2\gamma 2$ GABA_A receptors.

GABA _A R Configuration	GABA EC50		CSP gating (% of GABA max)		CSP modulation (% of GABA EC20)	
	(μ M)	n	3mM	n	300 μ M	n
α 3 WT	34.8 ± 2.1	6	08.5 ± 1.1	11	235 ± 35	6
α 3(V440L)	07.5 ± 0.9 **	7	37.6 ± 3.5 **	9	301 ± 14	5
α 3(I419L/I423A)	18.1 ± 2.2 *	9	40.8 ± 2.4 **	8	252 ± 14	4
α 3(I419L/I423A/V440L)	15.8 ± 5.0 *	6	35.9 ± 3.9 **	10	156 ± 22	6

TABLE III-1

Table III-2. Influence of GABA_A receptor α 1 TM4 residues mutation to corresponding α 3 amino acids and α 1 (L415) amino acid substitution on GABA EC50 values and carisoprodol direct activation properties. Carisoprodol direct gating activation is normalized to saturated GABA current whereas carisoprodol modulation effect to potentiate GABA-gated current is normalized to GABA EC20 current. Each data point represents the mean \pm S.E.M of n cells. *, $p < 0.05$; **, $p < 0.01$ relative to wild type α 1 β 2 γ 2 GABA_A receptors.

GABA _A R Configuration	GABA EC50		CSP Direct gating (% of GABA max)		
	(μ M)	n	Maximum Efficacy	EC50(μ M)	n
α 1 WT	35.5 \pm 0.6	5	41.8 \pm 2.4	685 \pm 32	15
α 1(L394I)	50.8 \pm 4.4	4	40.6 \pm 4.6	559 \pm 50	6
α 1(A398I)	35.2 \pm 4.1	5	36.1 \pm 2.9	617 \pm 83	6
α 1(L415V)	89.0 \pm 2.2**	5	17.7 \pm 3.1**	826 \pm 24	9
α 1(L394I/A398I)	27.0 \pm 3.4	3	42.0 \pm 5.7	443 \pm 28	9
α 1(A398I/L415V)	68.4 \pm 4.2 **	3	22.6 \pm 1.8**	456 \pm 40	5
α 1(L394I/L415V)	27.5 \pm 3.1	3	20.5 \pm 3.2**	864 \pm 21	9
α 1(L394I/A398I/L415V)	44.1 \pm 2.4	7	18.2 \pm 4.5**	380 \pm 20	7
α 1(L415C)	39.4 \pm 4.0	4	18.0 \pm 1.6**	697 \pm 41	9
α 1(L415W)	47.7 \pm 2.3	3	17.8 \pm 4.1**	2056 \pm 122**	6
α 1(L415G)	68.0 \pm 4.3**	4	09.1 \pm 1.6**	545 \pm 10	5
α 1(L415S)	65.0 \pm 4.2**	7	05.5 \pm 1.2**	651 \pm 24	13
α 1(L415T)	41.0 \pm 2.1	4	37.1 \pm 4.4	533 \pm 41	9
α 1(L415Y)	45.0 \pm 3.2	4	25.4 \pm 3.8	807 \pm 55	6
α 1(L415I)	40.3 \pm 2.2	4	31.9 \pm 4.1	450 \pm 30	7
α 1(L415R)	43.4 \pm 5.2	4	32.6 \pm 4.6	492 \pm 24	4

TABLE III-2

Figure III-1. GABA_A receptor structure and alignment of transmembrane 4 amino acid residue of α (1-6) GABA_A receptor subunits. **A**, Top view of GABA_A receptor, representing arrangement of α 1, β 2 and γ 2 subunits and GABA and benzodiazepine (BDZ) binding sites. **B**, Lateral view of GABA_AR subunit illustrating, 4 transmembrane domains, the extracellular N-terminus, the C-terminus and the intracellular loop. **C**, Aligned Amino acid sequence of TM4 region of human α subunit (α 1-6) isoforms showing conserved (*) and non-identical (red) amino acids.

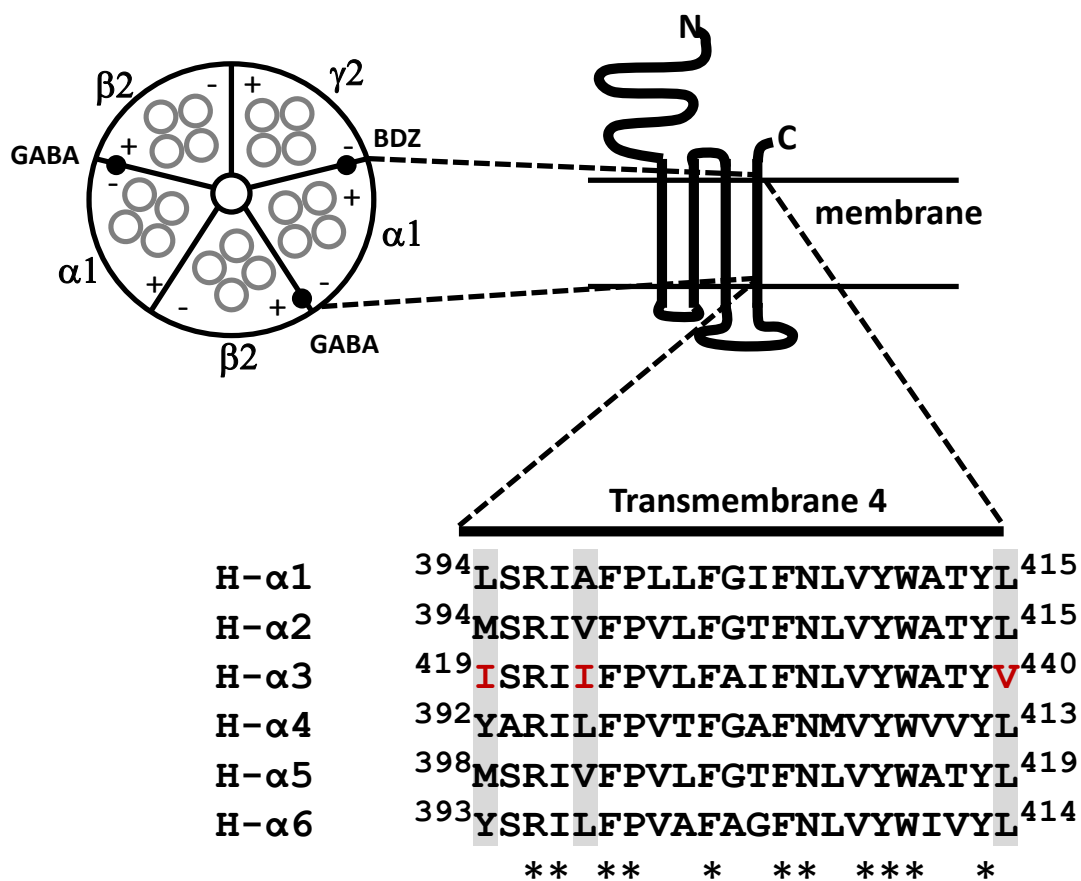


FIGURE III-1

Figure III-2. Influence of $\alpha 3$ subunit TM4 residues mutation on direct activation and allosteric modulation by carisoprodol. **A**, representative traces demonstrating carisoprodol (CSP) activates human $\alpha 3\beta 2\gamma 2$ WT and $\alpha 3(V440L)\beta 2\gamma 2$ GABA_ARs. Single mutation of V440 at TM4 of $\alpha 3$ to L, which is present in $\alpha 1$ -expressing GABA_ARs, increased CSP direct gating potency significantly. **B**, bar graphs summarizing carisoprodol direct gating currents for human $\alpha 3$ -, $\alpha 3(V440L)$ -, $\alpha 3(I419L/I423A)$ -, $\alpha 3(I419L/I423A/V440L)$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs. Single and combined mutation of TM4 domains of $\alpha 3$ to those present in $\alpha 1$ subunit significantly increased the direct gating potency of carisoprodol as compared to WT $\alpha 3$ receptors. Carisoprodol-gated currents are normalized to currents elicited by saturated GABA concentration (1 mM). **C**, representative traces demonstrating the potentiation of GABA-gated (EC₂₀) currents from human $\alpha 3\beta 2\gamma 2$ WT and $\alpha 3(V440L)\beta 2\gamma 2$ GABA_ARs by carisoprodol. **D**, concentration-response curves for the allosteric modulation of GABA-gated currents from human $\alpha 3$ -, $\alpha 3(V440L)$ -, $\alpha 3(I419L/I423A)$ -, $\alpha 3(I419L/I423A/V440L)$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs. Mutation of TM4 domains of $\alpha 3$ to those present in $\alpha 1$ subunit did not increased allosteric modulation efficacy of carisoprodol significantly as compared to WT $\alpha 3$ receptors. Carisoprodol potentiated currents are normalized to currents elicited by GABA EC₂₀ concentrations. Each data point represents the mean \pm S.E.M. of a minimum of three cells. **, $p < 0.01$, *, $p < 0.05$.

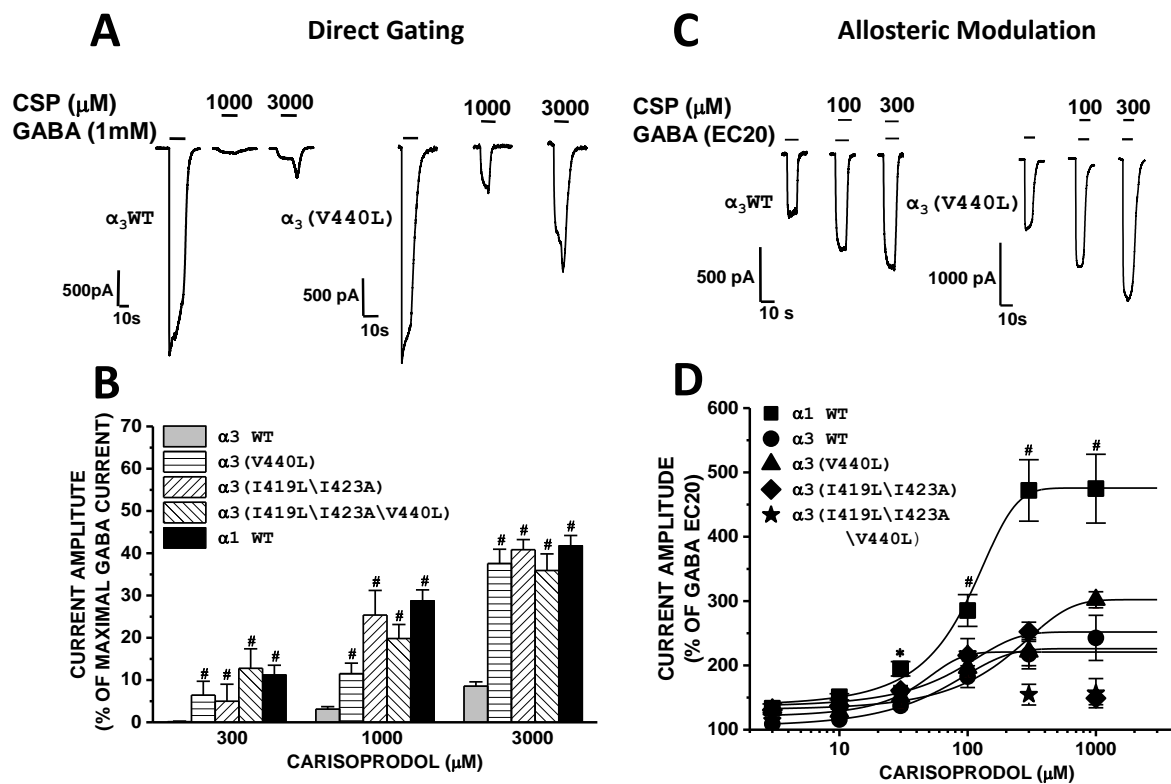


FIGURE III-2

Figure III-3. Influence of the $\alpha 1$ subunit TM4 residues mutation on direct activation by carisoprodol. **A**, representative traces demonstrating carisoprodol (CSP) activates human $\alpha 1\beta 2\gamma 2$ WT and $\alpha 1(L415V)\beta 2\gamma 2$ GABA_ARs. Corresponding reverse mutation of L415 at TM4 of $\alpha 1$ to V (present in the $\alpha 3$ subunit) decreased carisoprodol direct gating potency significantly, confirming involvement of TM4 residue L415 in direct gating. **B**, bar graphs summarizing carisoprodol direct gating currents for human $\alpha 3$ -, $\alpha 1(L394I)$ -, $\alpha 1(A398I)$ -, $\alpha 1(L415V)$ -, $\alpha 1(L394I/A398I)$ -, $\alpha 1(L394I/L415V)$ -, $\alpha 1(A398I/L415V)$ -, $\alpha 1(L394I/A398I/L415V)$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs. All $\alpha 1$ - GABA_ARs containing L415V mutation showed decreased CSP direct gating potency as compared to WT $\alpha 1\beta 2\gamma 2$ GABA_ARs. Carisoprodol-gated currents are normalized to currents elicited by saturated GABA concentration (1mM). Each data point represents the mean \pm S.E.M. of a minimum of three cells. **, $p < 0.01$.

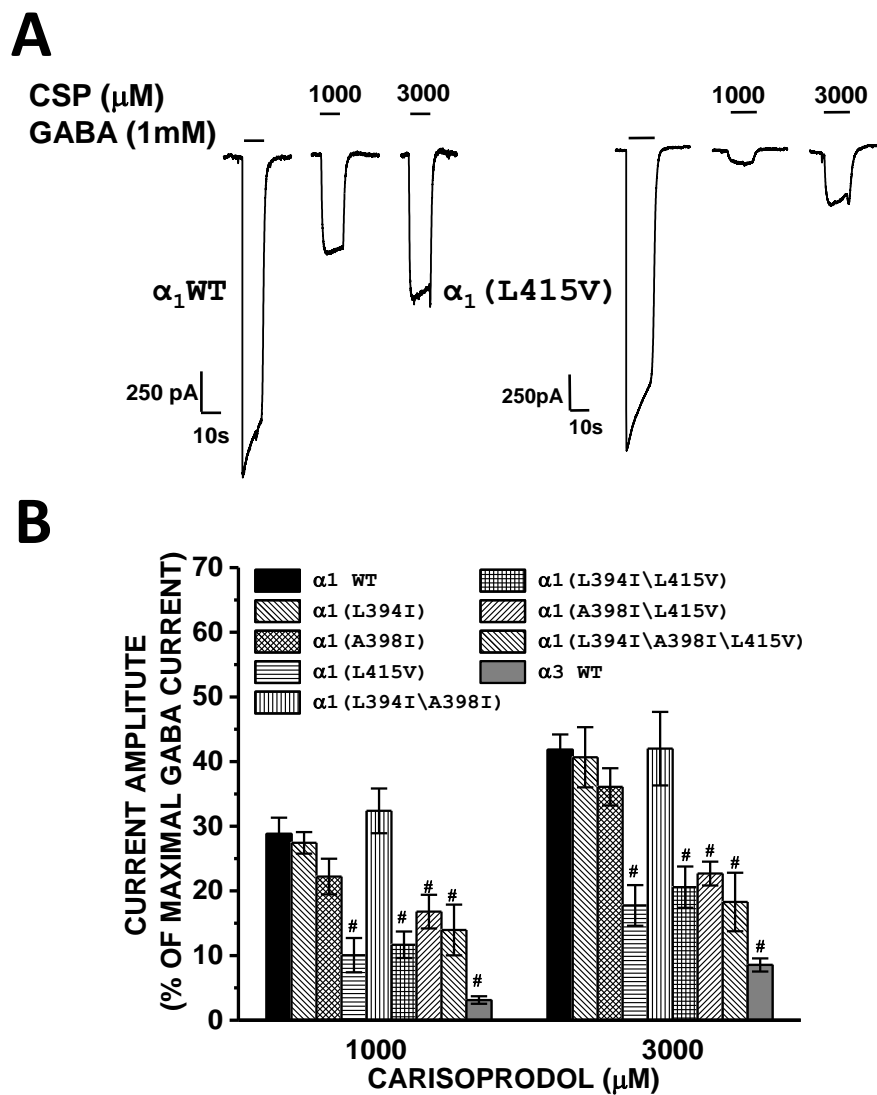


FIGURE III-3.

Figure III-4. Influence of the $\alpha 1$ subunit TM4 L415 mutations on carisoprodol direct gating potency and efficacy. **A**, representative traces demonstrating carisoprodol (CSP) activates human $\alpha 1(\text{L415S})\beta 2\gamma 2$ GABA_ARs. Carisoprodol reached saturation at 5 mM concentration and blocking effects started becoming prominent. **B**, concentration-response curves for the direct gating effect of carisoprodol from human $\alpha 1(\text{L415S})$ -, $\alpha 1(\text{L415V})$ -, $\alpha 1(\text{L415G})$ -, $\alpha 1(\text{L415W})$ -, $\alpha 1(\text{L415C})$ - and $\alpha 1\beta 2\gamma 2$ GABA_ARs. These 5 mutations all decreased carisoprodol efficacy significantly without affecting carisoprodol EC₅₀, except for $\alpha 1(\text{L415W})$ mutant which increased EC₅₀ 3-fold relative to wild type receptors. **C**. Bar graphs summarizing carisoprodol (CSP) direct gating currents for human $\alpha 1$ WT-, $\alpha 1(\text{L415I})$ -, $\alpha 1(\text{L415T})$ -, $\alpha 1(\text{L415R})$ -, $\alpha 1(\text{L415Y})$ -, $\alpha 1(\text{L415W})$ -, $\alpha 1(\text{L415V})$ -, $\alpha 1(\text{L415C})$ -, $\alpha 1(\text{L415G})$ -, $\alpha 1(\text{L415S})\beta 2\gamma 2$ GABA_ARs. Carisoprodol-gated current reached saturation at 5 mM; receptors containing $\alpha 1$ (L415T/R/Y/I) mutant residue did not affect carisoprodol direct gating efficacy and thus carisoprodol EC₅₀ values were not calculated. Carisoprodol gated currents are normalized to currents elicited by saturated GABA concentration (1 mM). Each data point represents the mean \pm S.E.M of a minimum of three cells. **, $p < 0.01$.

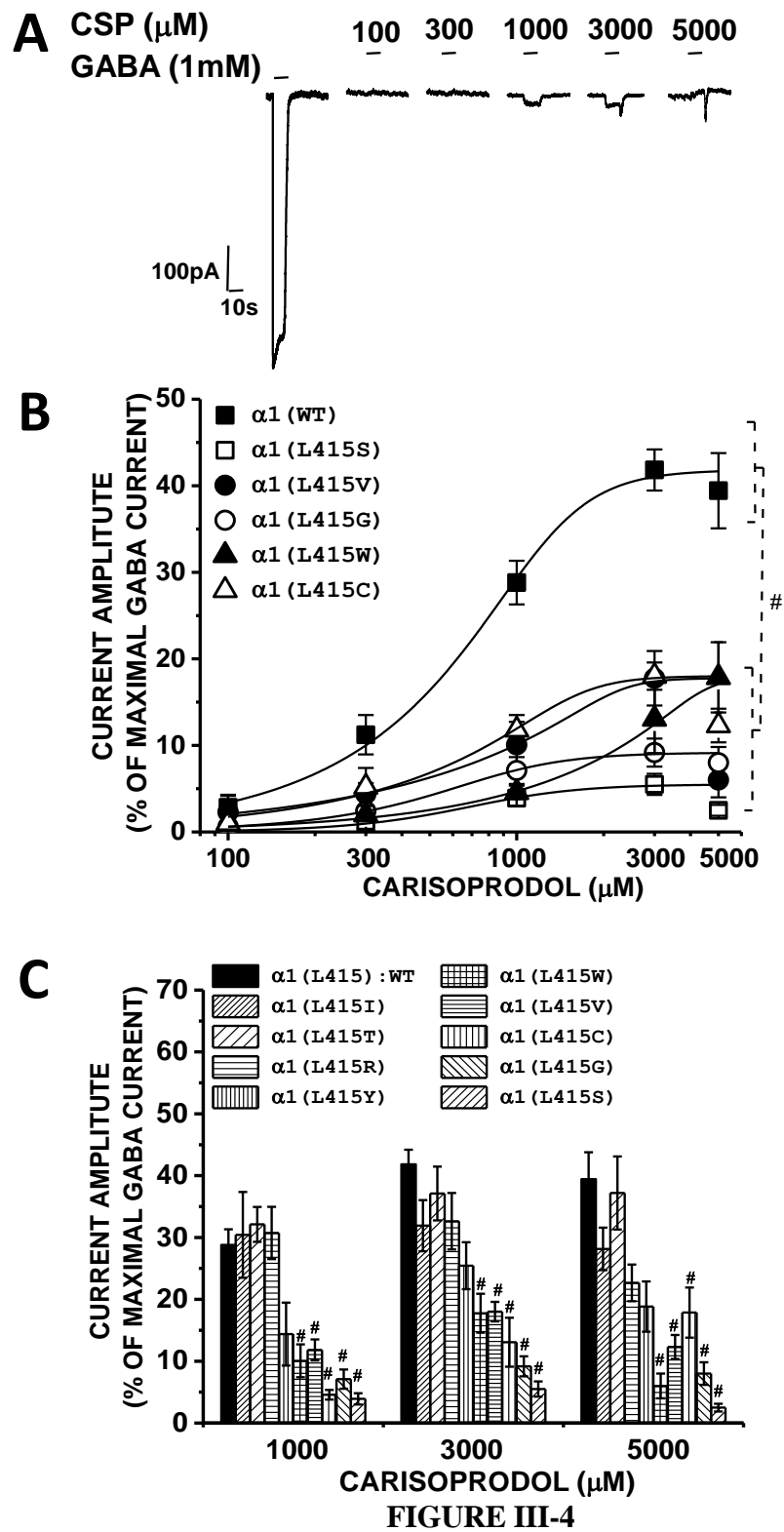


Figure III-5. Physiochemical relation of $\alpha 1$ 415 residue with carisoprodol direct gating efficacy, GABA EC₅₀ and GABA EC₅₀ with carisoprodol efficacy. Correlations with carisoprodol efficacy with $\alpha 1(415)$ position amino acids **A**, Hydropathy; **B**, Volume; **C**, Polarity. Correlations with GABA EC₅₀ with amino acids **D**, Volume and **E**, Hydropathy; **F**, Correlations of $\alpha 1$ mutant GABA EC₅₀ with carisoprodol efficacy. Both amino acid volume and polarity at the 415 position were correlated with carisoprodol efficacy. Each data point represents the mean \pm S.E.M of a minimum of three cells. *, $p < 0.05$.

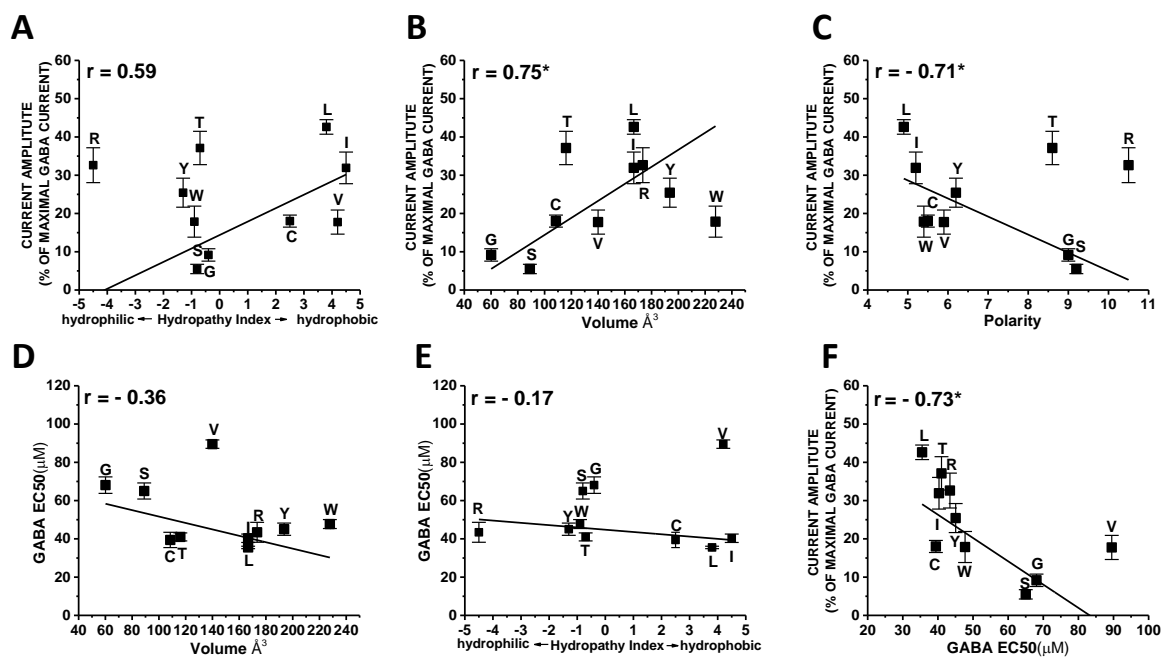


TABLE III-5.

Figure III-6 Influence of the alpha1 subunit TM4, L415S mutation on allosteric modulation by carisoprodol and pentobarbital direct activation. **A**, representative traces demonstrating the potentiation of GABA-gated (EC_{20}) currents from human $\alpha 1(L415S)\beta 2\gamma 2$ GABA_ARs by carisoprodol (CSP). **B**, concentration-response curves for the allosteric modulation of GABA-gated currents from human wild type $\alpha 1\beta 2\gamma 2$ and $\alpha 1(L415S)\beta 2\gamma 2$ GABA_ARs. Mutation of leucine to serine at $\alpha 1(415)$ residue, did not affect allosteric modulation by carisoprodol, confirming the amino acid at position 415 is solely involved in the direct gating effects. Carisoprodol-potentiated currents are normalized to currents elicited by GABA EC_{20} concentration. **C**, representative traces demonstrating pentobarbital (1mM) activation of human $\alpha 1(L415S)\beta 2\gamma 2$ GABA_ARs. **D**, bar graphs summarizing pentobarbital direct gating currents for human wild type $\alpha 1\beta 2\gamma 2$ and $\alpha 1(L415S)\beta 2\gamma 2$ GABA_ARs. Mutation of leucine to serine at $\alpha 1(415)$ residue, did not affect direct activation by pentobarbital on GABA_ARs. Pentobarbital-gated currents are normalized to the currents elicited by saturating concentration of GABA (1 mM). Each data point represents the mean \pm S.E.M. of a minimum of three cells.

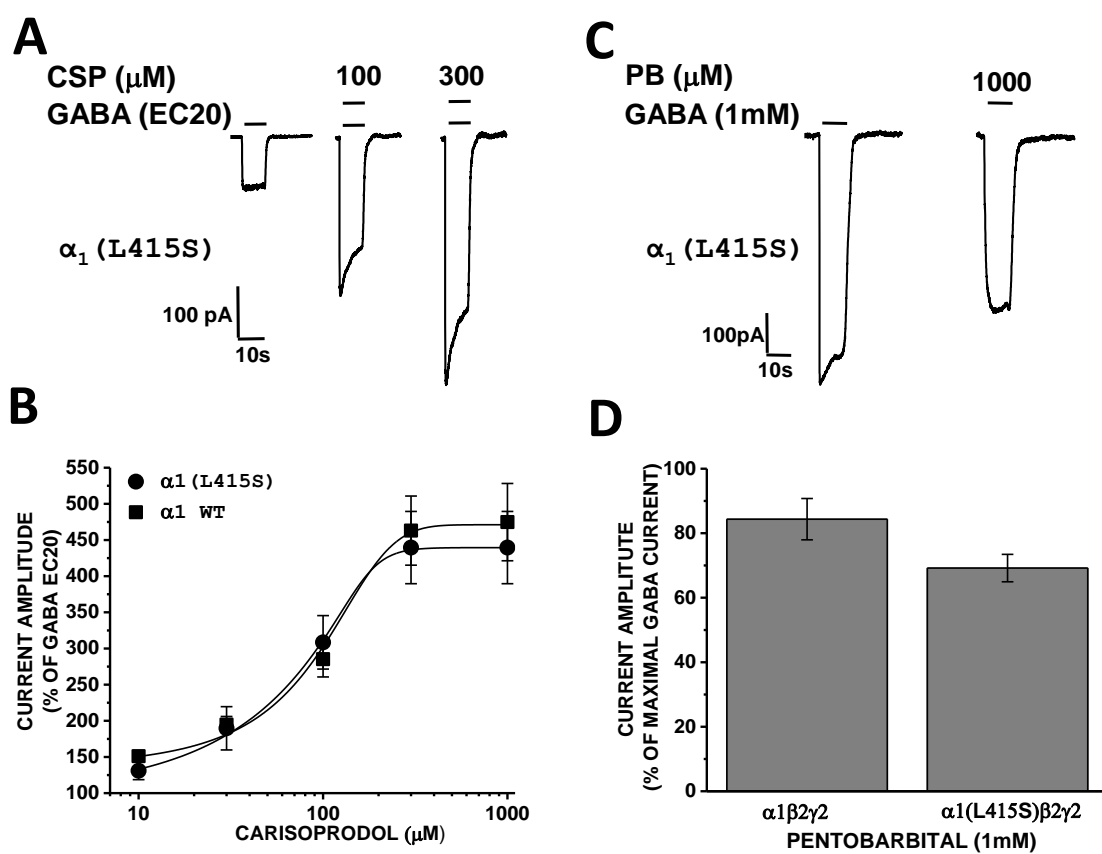


FIGURE III-6

Figure III-7. Homology molecular model of GABA_A receptor. A, Side view illustrating alpha subunits in red, beta subunits in blue and gamma subunit in yellow. B, Highlighted residue leucine 415 of $\alpha 1$ subunit, potential amino acid involved in direct gating action of CSP.

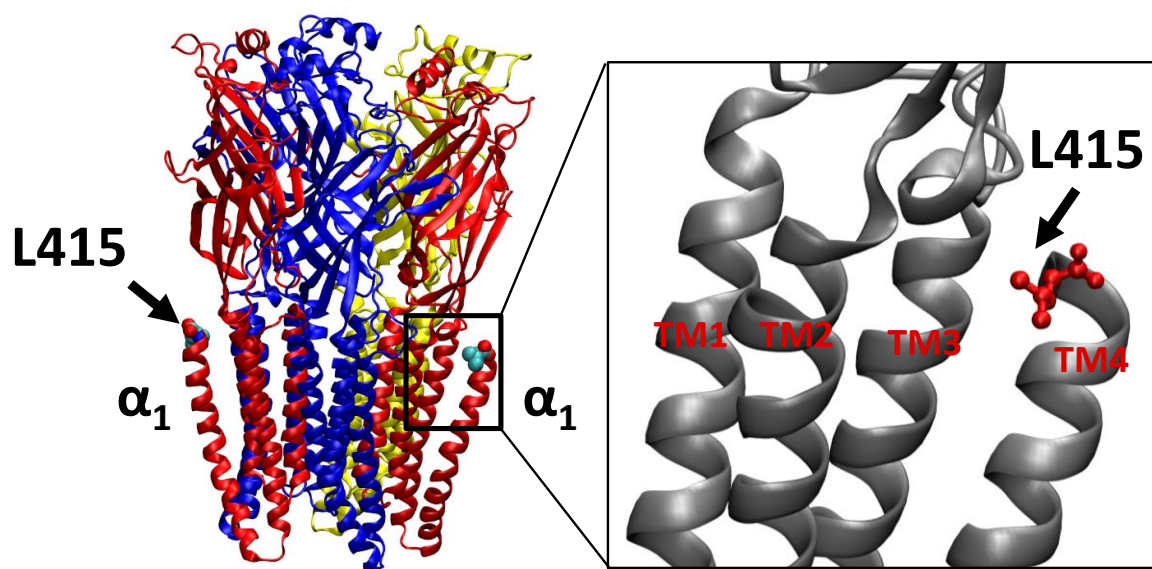


FIGURE III-7

AUTHORSHIP CONTRIBUTIONS

Participated in research design: **Kumar and Dillon**

Conducted mutagenesis: **Kumar and Freund**

Conducted experiments: **Kumar**

Performed data analysis: **Kumar and Dillon**

Wrote or contributed to writing of the manuscript: **Kumar and Dillon**

Footnotes

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CHAPTER IV

SUMMARY AND DISCUSSION

Carisoprodol is a centrally-acting skeletal muscle relaxant frequently prescribed for acute musculoskeletal conditions (Elder, 1991). Recreational use of carisoprodol is an increasing problem (Bramness et al., 2004; Bramness et al., 2007; Ni et al., 2007). Considering its alarming rate of abuse and subsequent consequences, carisoprodol was scheduled (schedule IV) at the federal level effective January 11, 2012 (DEA, 2011). Given the present and potential dangers posed by carisoprodol abuse, it is of crucial importance to determine how this drug mediates its effects. Until recently, it was widely accepted that the sedative and muscle relaxant effects of carisoprodol were due predominantly to its metabolite, meprobamate. However, it is now clear that carisoprodol itself modulates and directly gate GABA_ARs. Thus, our studies were aimed at contributing to the current knowledge regarding the mechanism of action of carisoprodol as a therapeutic agent and as a drug of abuse.

Subunit-dependent studies (Chapter II) have shown carisoprodol's actions on GABA_A receptors are influenced by subunit isoforms present in a receptor. Carisoprodol is most efficacious on $\alpha 1$ subunit containing GABA_A receptors for potentiating the GABA-gated currents. This may underlie the ability of carisoprodol in enhancing the sedative effects of CNS depressants, contributing to its potential for abuse. The direct gating action of carisoprodol on GABA_A receptor is generally comparable regardless of α subunit isoform, however, receptors incorporating the $\alpha 3$ subunit showed significantly lower direct gating

efficacy and affinity. Presence or absence of the $\gamma 2$ subunit did not affect the ability of carisoprodol to directly gate or allosterically modulate the receptor. Regarding β subunit isoforms, incorporation of $\beta 1$ subunit conferred highest efficacy for direct activation relative to maximum GABA currents, while presence of the $\beta 2$ subunit conferred highest efficacy for allosteric modulation of the GABA response. In δ subunit expressing extrasynaptic $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ receptors, carisoprodol is more efficacious than GABA as a direct gating agonist and allosterically potentiated both saturated and sub-saturating GABA currents. We also studied the voltage-dependent effect of carisoprodol on GABA_A receptors. We found, allosteric modulatory effect of carisoprodol is voltage sensitive while direct gating effect is voltage independent. These results suggest carisoprodol interacts with distinct sites to allosterically modulate and directly gate the GABA_AR, and subunit isoform influences these effects. As mentioned earlier, physical dependence is more prone to develop with drugs that interact with a broader collection of GABA_AR subtypes (Licata and Rowlett, 2008), the high efficacy of carisoprodol for both direct and allosteric effects on $\alpha 1$ -containing receptors, coupled with its ability to interact with GABA_AR subtypes, is consistent with its high abuse potential (Rudolph and Knoflach, 2011).

After gaining information regarding carisoprodol's GABA_AR subunit selectivity, we identified the subunit amino acid domains involved in direct gating and allosteric modulation effects of carisoprodol (Chapter III). To gain insight into the molecular mechanism of action of carisoprodol and specific amino acid domains involved in the direct gating and allosteric modulatory effects of carisoprodol, we aligned the amino acid sequence

of α subunit isoforms and took advantage of site directed mutagenesis. We identified several residues in TM4 of the $\alpha 3$ subunit that are unique compared to residues present in all other α subunits. We tested their involvement in the ability of carisoprodol to directly gate the GABA_AR by mutating these residues to those present in the $\alpha 1$ subunit, individually and in combination, and assessing effects on carisoprodol elicited gating. In single and combined mutations expressing the $\alpha 3(V440L)$ residue mutation, carisoprodol gained significant capacity to directly gate the receptor compared to $\alpha 3$ WT receptors. In contrast, the V440L mutation did not affect carisoprodol's ability to allosterically modulate GABA-activated currents. These results demonstrate amino acid residues in TM4 of the GABA_A receptor are critical in the direct gating, but not allosteric modulatory actions of carisoprodol

To confirm the involvement of TM4 residues in carisoprodol direct gating, we did corresponding reverse mutations in $\alpha 1$ subunit, individually and in combination, and tested to see if these mutations decrease carisoprodol sensitivity of $\alpha 1\beta 2\gamma 2$ receptors. In single and combined mutations that included the TM4 L415 residue, direct gating activity of carisoprodol was significantly reduced. Specifically carisoprodol direct gating efficacy reduced to 18% of $\alpha 1(L415V)\beta 2\gamma 2$ receptors from 42% of WT $\alpha 1\beta 2\gamma 2$ receptors. To further investigate the role of the $\alpha 1$ TM4 415 region, we substituted the wild type leucine with eight different amino acids one by one based on size, volume, polarity, hydrophobicity and hydrophobicity to study the effect of physio-chemical properties of amino acid side chains on carisoprodol direct gating affinity and efficacy. Insertion of isoleucine, arginine, tyrosine and threonine at $\alpha 1$ 415 position did not affect carisoprodol direct gating efficacy,

however, insertion of cysteine, tryptophan, valine, glycine and serine significantly decreased the direct gating efficacy relative to wild type. Interestingly, substitution of leucine with serine decreased direct gating efficacy to 5.5% from 42% (WT) but insertion of threonine did not affect carisoprodol direct gating efficacy suggesting orientation of hydroxyl and isopropyl group at $\alpha 1$ 415 position plays a critical role in carisoprodol direct gating efficacy. In physio-chemical analysis of substituted amino acid side chain properties with carisoprodol's direct gating efficacy, we concluded that the volume and polarity of amino acid side chain at 415 position of $\alpha 1$ subunit significant affect carisoprodol direct gating efficacy. To further confirm the involvement of $\alpha 1$ (415) domain only in direct gating action and not in allosteric modulatory effect, we tested $\alpha 1$ (L415S) mutant for allosteric modulatory effect of carisoprodol and found insertion of serine at $\alpha 1$ (415) position does not affect the allosteric modulatory effect of carisoprodol. The $\alpha 1$ (L415S) mutation also did not affect pentobarbital direct gating efficacy. Thus, $\alpha 1$ (L415) amino acid residue is solely involved in the direct gating effect of carisoprodol but not in the allosteric modulation. These data confirmed carisoprodol interacts with distinct sites to allosterically modulate and directly gate the GABA_A receptors.

The central nervous system adapts to its ambience constantly and it is not surprising that prolonged exposure to drug molecules elicits compensatory changes at the receptor level. A single intoxicating dose of alcohol results in down regulation of $\alpha 4\beta\delta$ receptors in hippocampal pyramidal neurons leading to generation of acute tolerance (Hemby et al., 2006; Shen et al., 2011). With a few more doses of alcohol, internalization of $\alpha 1$ subunit receptors

at synapses occurs, leading to mobilization of $\alpha 4$ subunit receptors to the synapses from extrasynaptic region. Down regulation of $\alpha 4\beta\delta$ receptors at extrasynaptic region is compensated by overexpression of $\alpha 4\beta\gamma$ receptors to supplement tonic inhibition (Liang et al., 2007). This mobilization of receptors is reversible, however, during chronic consumption of alcohol it becomes irreversible resulting in alcohol dependence (Liang et al., 2007). With chronic opiate exposure and following withdrawal, GABA_ARs signaling transition from inhibitory to excitatory in ventral tegmental area, contributing to opiate dependence (Laviolette et al., 2004). Carisoprodol abuse is also associated with dependence, tolerance, and withdrawal (Heacock and Bauer, 2004; Reeves et al., 2012; Reeves et al., 2007). We did not explore the changes associated with chronic carisoprodol administration, but it is likely that carisoprodol elicits fundamental changes in the GABAergic system. Moreover, chronic administration of drugs that target GABA_ARs reorganize the GABAergic system transmission and may lead to tolerance; the extent of modification depends upon the dose and duration of drug exposure (Korpi et al., 2002; Wafford, 2005). This event may be due to uncoupling of allosteric sites (Ito et al., 1996), modifications in receptor turnover (Kumar et al., 2003; Pericic et al., 2003), or desensitization. Whether expression of a subunit is upregulated or down regulated in response to chronic use varies with its location in the brain (Wafford, 2005). In some studies, down regulation of the $\alpha 1$ subunit has been observed, which is generally accompanied by compensatory up regulation of other subunits (Ito et al., 1996; Wafford, 2005). We have shown carisoprodol is most efficacious on $\alpha 1$ -containing receptors and less efficacious on other receptor configurations, displacing $\alpha 1$ subunits with

other isoforms may diminish the physiological effects of carisoprodol. The shift towards GABA_AR configurations that are less sensitive to carisoprodol's effects may contribute to tolerance. Such compensatory mechanisms associated with chronic activation of the GABAergic system are analogous to inhibitory dysregulation. Thus, abrupt cessation of the drug is likely to precipitate withdrawal symptoms as the central nervous system attempts to restore normal inhibitory function.

While carisoprodol remains an efficacious choice for the treatment of acute low back pain, its abuse liability has become a major concern in recent years, and ready availability and low cost has made it a drug of choice among abusers. Scheduling of carisoprodol in Norway resulted in a rapid and significant decline in the detection of carisoprodol in autopsies and driving under the influence occurrences (Hoiseth et al., 2009). As carisoprodol is now controlled in the United States at the federal level, it is likely the incidence of abuse and fatalities will also decline here. Physician awareness regarding potential dangers associated with abuse of carisoprodol and its metabolite meprobamate should also improve with the recent scheduling. However, considering the fact that low back pain has been one of the top five reasons for physician visits in the United States for decades (Witenko et al., 2014), the need to develop a safe and efficacious agent for this substantial medical need is clear. Also, while the number of reports regarding carisoprodol abuse continues to increase, there has been little progress in the treatment of carisoprodol dependence and withdrawal. At present, treatment consists of brief courses with benzodiazepines or phenobarbital to combat anxiety and insomnia. Further, treatment of

carisoprodol overdose is complicated as it is often characterized by agitation and seizures, and the administration of anticonvulsants and sedatives exacerbates CNS depression, leaving supportive therapy as a preferred course of action. The insights gained in our study of carisoprodol's molecular action should prove useful in better understanding how to treat misuse, and in development of improved muscle relaxants with reduced abuse liability.

FUTURE DIRECTIONS

In the present study we concluded that distinct amino acids or binding sites are involved in direct gating and in the allosteric modulation action of carisoprodol. We identified the leucine 415 residue at TM4 of $\alpha 1$ subunit is critical in the direct gating action of carisoprodol. Docking of carisoprodol on a molecular model of GABA_A receptors will provide further insights into the binding pocket amino acids of carisoprodol. However, it is still unclear whether L415 is involved in the binding pocket of carisoprodol of GABA_A receptors or if it is involved in the conformational wave leading to channel gating called coupling. Ligand binding assays, single channel analysis, and substituted cysteine accessibility method (SCAM) experiments at mutant $\alpha 1(L415S)\beta 2\gamma 2$ GABA_A receptors would clarify the importance of this residue in carisoprodol's direct gating action. Single channel (open probability) analysis would also provide critical information on whether channel opening gated by carisoprodol is more like pentobarbital (long duration opening) or benzodiazepines (burst openings) (MacDonald et al., 1989; Steinbach and Akk, 2001; Thompson et al., 1999).

It cannot be denied that the allosteric modulatory effect of carisoprodol is highly important in physiological actions of carisoprodol and we have shown that carisoprodol is highly efficacious at potentiating GABA-gated currents. In the present study, that we did not identify the molecular domains involved in the allosteric modulatory effect of carisoprodol. However, we demonstrated that carisoprodol is highly efficacious on $\alpha 1$ subunit containing receptors compared as to other isoforms of α subunit and the fact the $\alpha 1$ subunit GABA_A

receptors are involved in the addiction and sedative mechanisms of benzodiazepines (Ator, 2005; Tan et al., 2010). It is very critical to determine the molecular mechanism of allosteric modulation by carisoprodol. We have identified two amino acids in the pre-TM1 region (G200 and V202) and one amino acid at the N- terminal (F45) region of $\alpha 1$ subunit which are unique compared to the rest of the α subunit isoforms in the extracellular domains of GABA_A receptor. Investigation of these amino acids may provide critical information about carisoprodol's allosteric modulation binding site.

Our study demonstrates that GABA_A receptor subtype isoforms influence carisoprodol's action and the molecular sites where carisoprodol can interact to modulate GABA_A receptor functions. Replication of our experiments in an in-vivo model would allow us get complete knowledge of carisoprodol's subunit-dependent physiological effects. Therefore, it is critically important to investigate which of the GABA_AR subunit isoforms account for specific behavioral actions of carisoprodol. The extensively used techniques to study specific GABA_AR subunit isoform with behavior are genetic deletion of a subunit isoform in a knockout mouse and viral delivery of inhibitory RNAs or other RNAs to generate knockdown in specific brain regions. The knockout model of specific GABA_A receptor subunits have been studied for alcohol research (Blednov et al., 2012). The $\alpha 1$ and $\alpha 2$ subunit knockin mice showed $\alpha 1$ GABA_A receptors are involved in alcohol tolerance and physical dependence while $\alpha 2$ GABA_A receptors with taste aversion, alcohol consumption, and motor stimulation (Hemby et al., 2006; Liang et al., 2007; Shen et al., 2011). Knockdown of $\alpha 4$ subunit together with δ subunit in the nucleus accumbens region

of the mice brain have been shown to reduce the alcohol intake, suggesting a critical role of extrasynaptic $\alpha 4\beta\delta$ GABA_A receptors in alcohol intake reinforcement (Nie et al., 2011). As mentioned earlier, behavioral effects of benzodiazepines have also been studied in mutant mice, where diazepam was made insensitive on specific GABA_A subunit isoforms by mutating arginine to histidine at its conserved site of action on $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 5$ subunit containing receptors (Rudolph et al., 1999). Diazepam's sedative, amnesic and anticonvulsant effects were absent in $\alpha 1$ (H101R) mutant mice. Mutant mice containing $\alpha 2$ (H101R) receptors showed absence of anxiolytic, motor-impairing and partly myorelaxant effects while retaining sedative effects of diazepam. Also, it's been shown that development of tolerance to the sedative effects of diazepam in mice requires $\alpha 5$ GABA_A subunit receptors (van Rijnsoever et al., 2004). Further, $\alpha 1$ subunit containing GABA_A receptors have been shown to be involved in addictive properties of benzodiazepines (Tan et al., 2010). GABA_A receptor α subunit isoforms knockout mice would provide importance of each α GABA_AR subunit isoform in carisoprodol's pharmacological effects. Also, $\alpha 1$ (L415S) knock in mutant mice will give insights into the role of the direct gating action of carisoprodol.

The ultimate goal of our study is to get critical information which will help in the development of a better muscle relaxant with reduced or no abuse potential. GABA_A receptor subunit-selective compounds have been developed and studied widely. Zolpidem (brand name Ambien) is a frequently prescribed sedative for sleep disorder that is selective for $\alpha 1$ GABA_A receptors (Rowlett et al., 2005). L-838417, a partial agonist at $\alpha 2$, $\alpha 3$ and $\alpha 5$ and antagonist at $\alpha 1$ GABA_A was developed as an analog of benzodiazepines as a non-sedative

anxiolytic (Rowlett et al., 2005). However, pharmacokinetic properties of this compound were not promising (Scott-Stevens et al., 2005). As mentioned previously, a drug selective primarily for α_2 -expressing GABA_A receptors would likely have less abuse potential and fewer sedative effects (Rudolph and Knoflach, 2011). SL651498 is a selective agonist of α_2 and α_3 GABA_A receptors and partial agonist at α_1 and α_5 GABA_A receptors, which is under development as non-sedative anxiolytic and muscle relaxant (Griebel et al., 2003; Whiting, 2006). As mentioned earlier, carisoprodol was developed as an analog of meprobamate (replacing isopropyl group on one of the carbamyl nitrogens) suggesting lower abuse liabilities and better muscle relaxation properties (Nebhinani et al., 2013) but recent evidence confirms carisoprodol high abuse potential (Reeves et al., 2012). However, subunit-dependence data has shown carisoprodol is more efficacious than meprobamate on GABA_A receptors (Kumar et al., 2012). These finding suggest carisoprodol would be a good candidate to investigate its structure-activity relationship on GABA_A receptor subunit isoforms.

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